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March 2005

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on Koi Herpesvirus Disease



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BULLETIN OF FISHERIES RESEARCH AGENCY

Supplement No. 2

March 2005

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Preface

In 1998, a shocking news on mass mortality of carp and koi was running into all over the world as reported from Israel, several European countries and the United State.

Soon, it revealed that the mass mortality of carp and koi was caused by a new specific viral disease so call as koi herpesvirus disease (KHVD). After that, it reported that the outbreak of KHVD was spread into Indonesia and Asian countries, and gave a great impact on carp and koi as a serious damage of mass mortality in both natural and cultured ones.

Though there was no reports for the outbreak of KHVD in Japan at that time, koi herpesvirus (KHV) was designated as a “Specific Disease” under the Law to Ensure Sustainable Aquaculture Production (LESAP) in 30 June 2003 because it could cause mass mortality of carp and koi when an outbreak is happen in Japan. And finally, the massive death of carp in Lake Kasumigaura and Kitaura of Ibaraki Prefecture in early October 2003 was observed. That was also the first case which the occurrence of KHVD was confirmed in Japan.

Under the state of emergency, the Ministry of Agriculture, Forestry and Fisheries (MAFF) requested every prefecture to detect KHVD. At the same time on-consignment of MAFF, Fisheries Research Agency (FRA) has conducted imminent research studies on tracing KHVD. And the National Research Institute of Aquaculture (NRIA), FRA, gave the guideline which provides etiologial information, symptom, diagnostic procedures by a polymerase chain reaction (PCR) test for KHV detection and other important characteristics of the disease through the technical training (seminar) which was held in NRIA due to learn PCR procedure for KHV detection. After that, PCR inspection for KHV infection was established in accordance with the chart in the guideline. Since then, the MAFF has shown its appropriate preventive measures against KHVD spread according to the LESAP. And the MAFF was also working on the Expert Council Meeting for checking and improving the current situation of quarantine system of aquatic products.

Under the current circumstance, the International Symposium on KHVD was held at 13 March 2004 in Pacifico Yokohama Japan with a collaboration of four organizations, the Fisheries Research Agency (FRA), the Southeast Asian Fisheries Development Center (SEAFDEC), the Fisheries Agency Japan (FAJ) and the World Organisation for Animal Health (OIE). Without their collaboration and support, this symposium could not be achieved.

In this symposium, the keynote lecture by Prof. Ronald P. Hedrick from the University of California, Davis, the U.S.A., a foremost expert on the disease provided variable information on the current status, measures for prevention and removal against KHVD. In addition, experts of various

countries from Europe, Asia and Japan in research and related field, gave together to disseminate and exchange the latest information on the disease and discuss on the control of KHVD for aiming appropriate preventive measures against its spread.

Sixteen papers were presented in the symposium. Ten full papers and five modified abstracts have been accepted for this Proceedings.

Finally I would like to give my most sincere thanks to the following four collaborative organizations for their greatly support together with their logo marks below.



Fisheries Research
Agency



Southeast Asian Fisheries
Development Center



Fisheries Agency
Japan



World Organisation for
Animal Health

Toshihiko Matsusato
Executive Director
Fisheries Research Agency

International Symposium on Koi Herpesvirus Disease
- Strategy for Koi Herpesvirus Disease Control -
(Saturday, 13 March 2004, Pacifico Yokohama Japan)

Kyoichi Kawaguchi
President, Fisheries Research Agency (FRA)

On behalf of Fisheries Research Agency (FRA), I, “Kawaguchi: President of FRA”, would like to give my most sincere welcome thanks to all participants to be here from Fisheries Agency, universities, and private concerned, especially to all scientists from foreign countries as well as Japan.

About middle of October 2003, the outbreak of mass mortality of carp in Lake Kasumigaura and Kitaoura of Ibaraki Prefecture was observed. Throughout scientific investigations, the first occurrence of koi herpesvirus disease (KHVD) was confirmed in Japan. As a consequence, KHVD was designated as a specific disease under the Law to Ensure Sustainable Aquaculture Production (LESAP) in June 2003.

And appropriate preventive measures against KHDV spread are now taken according to LESAP. But just it starts and further research for KHDV control will be hope to responsibility.

Under on-consignment of Ministry of Agriculture, Forestry and Fisheries (MAFF), FRA has conducted imminent research studies on tracing KHVD infection and its impact on common carp and koi. National Research Institute of Aquaculture (NRIA), FRA, gives the guideline which provides etiological information, symptom, diagnostic procedures and other important characteristics of the disease through the technical training (seminar) held in NRIA due to learn a polymerase chain reaction (PCR) procedure for koi herpesvirus (KHV) detection. After that, PCR inspection for KHV infection was established to do in accordance with the chart in the guideline. Detailed report will be presented later by NRIA's scientists.

Meanwhile outbreaks of KHVD in Israel, United State of America, Europe and Indonesia etc., give a serious impact on common carp and koi as shown of mass mortality. The present situation of KHVD impact will be reported here by all scientists from various countries as invited speakers. I am great gratitude and hope for very valuable information will be given in this symposium.

For a proper management measures against KHVD, it is necessary to grasp current situation of KHVD infection at first, and then to develop the preventive measures against KHVD. To achieve this purpose, this symposium will provide and disseminate the latest information about KHVD impacts from various countries. And it is primary important that we should discuss further research activities

on the base of common knowledge about KHVD. This International Symposium is organized for aiming appropriate preventive measure against KHVD spread.

We can hold this symposium under the guidance of the Fisheries Agency Japan (FAJ), SEAFDEC, and the World Organisation for Animal Health (OIE). Without their collaboration and support, this symposium can not be achieved.

Finally, I would like to give my most sincere thanks to all organizations to be here, and also to express my hope that this symposium will help prevention and control of KHVD with the dissemination and exchange of information from all participants.

International Symposium on Koi Herpesvirus Disease
- Strategy for Koi Herpesvirus Disease Control -
(Saturday, 13 March 2004, Pacifico Yokohama Japan)

Junichiro Okamoto
Deputy Secretary-General,
Southeast Asian Fisheries Development Center (SEAFDEC)

It is a great honor for me to be here representing the SEAFDEC as a co-organizer of this international symposium on Koi Herpesvirus Disease (KHVD).

The SEAFDEC is an autonomous intergovernmental body established as a regional treaty organization in 1967 to promote fisheries development in South Asia. The member countries of SEAFDEC at present are Japan and 10 ASEAN countries. Four technical departments (Aquaculture, Fisheries resources research, Post-harvest processing and Training) and the Secretariat are established to pursue the objectives of SEAFDEC.

Aquaculture production has grown rapidly in last decade in Southeast Asia and has contributed much in food production and rural development in the region. However, infectious diseases are frequently found in aquaculture and have threatened the sustainability of aquaculture in Southeast Asia. Therefore, infectious disease has been one of serious concerns that the SEAFDEC has to tackle.

In Southeast Asia, KHVD was reported for the first time from Indonesia and Taiwan in 2002. The disease was also found in Japan in 2003, and KHVD has caused in high mortality of common and koi carp production in Indonesia and Japan. Common carp is an important food resources in rural areas of Southeast Asia, and koi carp is also internationally traded as valuable ornamental fish among countries. Under these situations, the SEAFDEC regards that KHVD is a newly emerging and serious threat to the common and koi carp aquaculture in Southeast Asia.

Since the year 2000, the Fish Disease Project has been implemented at the SEAFDEC as a Trust Fund Project sponsored by Government of Japan. Under this project, various researches and studies have been conducted on viral, bacterial and parasitic diseases of fish and shrimps by the SEAFDEC Aquaculture Department in Philippines in collaboration with Thailand and Singapore as well as Philippines.

The occurrence of KHVD has been confined so far to Indonesia in Southeast Asia. In order to prevent the spread of KHVD in other member countries, the SEAFDEC has started various activities for this serious

disease through implementation of Trust Fund Project “Fish Disease Project” sponsored by Japan. One of major activities in 2004 is research on KHVD. Fish Health Section’s staff of the SEAFDEC Aquaculture Department have a plan to conduct research and studies in the region. Another major activity is to co-organize and hold international meetings on aquatic animal disease. In addition to this symposium, the SEAFDEC will organize a two-day workshop in Manila, Philippines in this June in order to assess the current occurrence of transboundary fish disease in Southeast Asia and to find appropriate ways for development of fish disease quarantine and surveillance system in the region.

International collaboration and network is essential to prevent the spread of infectious diseases in the other countries. In this connection, the SEAFDEC will conduct various major activities for KHVD and other diseases in collaboration with other international organizations such as OIE and FAO.

In conclusion, I would like to give my most sincere thanks to the Government of Japan for her great support, especially through the Trust Fund for Fish Disease Project to the SEAFDEC.

I would like also to express my wishes that this symposium will help prevention and control of KHVD in Japan, SEAFDEC member countries and other countries.

International Symposium on Koi Herpesvirus Disease
- Strategy for Koi Herpesvirus Disease Control -
(Saturday, 13 March 2004, Pacifico Yokohama Japan)

Shiroh Yuge
Deputy Director-General of Fisheries Agency,
Ministry of Agriculture, Forestry and Fisheries

On behalf of Ministry of Agriculture, Forestry and Fisheries (MAFF), I would like to say a few words for “International Symposium on Koi Hepesvirus Disease.”

In 2000, it was reported that a new viral disease so call as koi herpesvirus disease (KHVD) caused mass mortality of carp and koi in Israel and the United States. Since then, outbreaks of KHVD in Europe and Indonesia have resulted in serious damages to both common carp and fancy koi carp (nishikigoi).

As for Japan koi herpesvirus was designated as a specific disease under the Law to Ensure Sustainable Aquaculture Production in June 2003 because it could cause mass mortality of carp and koi. While no outbreak of KHVD had been reported in Japan at that time, with the massive death of carp at Lake Kasumigaura and Kitaura in Ibaraki Prefecture in early October 2003, the occurrence of KHVD was confirmed for the first time in Japan.

MAFF requested every prefecture in Japan to detect KHVD and to take appropriate preventive measures against its spread according to the Law to Ensure Sustainable Aquaculture Production. In addition, MAFF holds a series of expert council meetings for reviewing and improving the current situation of quarantine systems for aquatic products.

Under this situation, it is my great pleasure to hold this International Symposium with all the participants including representatives from fisheries industries, in collaboration with Fisheries Research Agency (FRA), Southeast Asian Fisheries Development Center (SEAFDEC), and World Organisation for Animal Health (OIE).

In this symposium, the keynote lecture by Prof. Ronald P. Hedrick from the University of California, Davis, the U.S.A., a foremost expert on the disease will provide us with information on the current research

status, and measures for prevention and removal. In addition, experts in research and other related sectors from various countries in Europe and Asia, will have opportunities to disseminate and exchange the latest information on the disease and to discuss measures for control of KHVD.

Finally, I would like to give my sincere welcome to invited speakers from various countries around the world. I hope that this symposium will be fruitful not only for scientists but also all other participants.

International Symposium on Koi Herpesvirus Disease
- Strategy for Koi Herpesvirus Disease Control -
(Saturday, 13 March 2004, Pacifico Yokohama Japan)

Dr. Teruhide Fujita
Regional Representative for Asia and the Pacific
World Organisation for Animal Health (OIE)

Distinguished delegates and participants,
Distinguished speakers,
Ladies and gentlemen,

It is my great pleasure to be here with all the participants in this important International Symposium on Koi Herpesvirus Disease and to say a few words on behalf of Dr. Bernard Vallat, Director General of the World Organisation for Animal Health (in French acronym of OIE), Paris, France, and also on behalf of the OIE Regional Representation for Asia and the Pacific based in Tokyo, Japan.

OIE was established with its Headquarters in Paris, France in 1924 under the International Agreement signed by 18 countries at first, in order to promote world animal health.

OIE has now 167 member countries and its roles have been strengthened and expanded year by year to ensure transparency in the global animal disease situation; to collect, analyse and disseminate scientific veterinary information; to contribute expertise and encourage international solidarity in the control of animal diseases; to safeguard world trade by publishing health standards for international trade in animal and animal products, within its mandate under the World Trade Organization (WTO) SPS Agreement; in other words OIE is recognised by WTO as the international standards-setting organisation for animal health and zoonoses; to improve the legal framework and resources of Veterinary Services and to cope with its new mandates for animal production food safety and animal welfare.

OIE has been widely considered as an international and intergovernmental organisation for terrestrial animal health but has played important roles for aquatic animal health as well.

One of the OIE Specialist Commissions, namely the OIE Aquatic Animal Health Standards Commission, so-called “the Aquatic Animals Commission”, develops the *Aquatic Animal Health Code* to assure the sanitary safety of international trade in aquatic animals (fish, molluscs and crustaceans) and their products, and

provides a uniform approach to the diagnosis of diseases listed in the OIE *Aquatic Animal Health Code* so that the requirements for health certification in conjunction with trade in aquatic animals and aquatic animal products can be met.

Those OIE standards are continually revised and updated, and authorised by the OIE International Committee that is the highest governing body with participation of the OIE delegates from all the Member Countries, as new information on aquatic animal diseases in general and new emerging diseases in particular, becomes available.

Ladies and gentlemen,

The OIE HQ and its Regional Representation based in Tokyo are working with the Member Countries to collect and disseminate aquatic animal health information and for meetings including hands-on workshops on diagnosis and surveillance of aquatic animal diseases.

Aquatic animal diseases are quite important for the world, in particular in Asia as approximately 90% of world aquaculture production is covered by the Asian region.

Under these conditions, mass mortality of carp and koi was recognised in Israel and the United States in 1998, which included a new viral disease, Koi Herpesvirus (KHV) Disease. The disease was reported later also in Europe and the Asian region including Indonesia, and Japan in 2003, with a serious negative impact on the fisheries sector.

This International Symposium invites many internationally well-known scientists as speakers. And I do hope this meeting will discuss the control measures of KHV Disease and produce fruitful results of discussions which will no doubt contribute to the sound development of aquaculture not only in Asia but also in the world.

Thank you for your attention.

Initial Isolation and Characterization of a Herpes-like Virus (KHV) from Koi and Common Carp

Ronald P. HEDRICK, Oren GILAD, Susan C. YUN, Terry S. MCDOWELL,
Thomas B. WALTZEK, Garry O. KELLEY and Mark A. ADKISON

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Abstract

In September of 1998 our laboratory was asked to investigate epidemics characterized by high mortality occurring in populations of koi (*Cyprinus carpio*) in both the U.S.A. and Israel. Either live fish or frozen tissues arrived from both locations in September and November of 1998. In addition, tissues fixed for both light and electron microscopy from fish in Israel and the U.S.A. were obtained for examination. The virus observed and then isolated in 1998 from these initial samples has become the focus of a worldwide concern for the health and welfare of both captive and wild populations of common carp and ornamental koi. In this short report we review the initial isolation and characterization of the herpes-like virus referred to as koi herpesvirus or KHV. We also discuss the important role of temperature on in vivo and in vitro infections with the virus, recent comparisons of KHV to other herpes-like viruses from fish and lastly review current methods to detect the virus or evidence that fish have been exposed to the virus.

Key words: koi, common carp, koi herpesvirus, KHV, herpes-like viruses

Introduction

Outbreaks of a new disease among koi and common carp (*Cyprinus carpio*) may have occurred as early 1995 - 1996 but were more formally recognized in Germany in 1997 (Bretzinger *et al.*, 1999). The actual cause of the disease however, was not identified until 1998 following investigations of outbreaks among koi and common carp in Israel and the U.S.A. (Hedrick *et al.*, 1999, 2000). Those investigations demonstrated the presence of a herpes-like virus, designated koi herpesvirus or KHV, in diseased koi from Israel and the U.S.A. that was successfully isolated by use of a newly developed cell line from koi fin (KF-1). The isolated virus was demonstrated to induce the same disease and high mortality

characteristic of the natural outbreaks following experimental infections of koi in the laboratory (Hedrick *et al.*, 2000).

Several viruses have been isolated from koi undergoing high mortality and suffering from gill and skin diseases (Body *et al.*, 2000, Neukirch *et al.*, 1999, Oh *et al.*, 2001). However, the viruses in these reports clearly differ from KHV with respect to virion morphology, cytopathic effects (CPE) induced and types of cell lines that are susceptible, and the inability to cause disease upon experimental exposures of koi. Virus isolations that are similar or identical to KHV have been reported more recently by Neukirch and Kunz (2001), Ronen *et al.* (2003) and Perelberg *et al.* (2003) with the latter two reports preferring the designation for the agent as carp nephritis and gill necrosis virus (CNGV).

KHV has presumably spread through the largely unregulated movements of koi, although other unknown mechanisms may also be involved. The virus now has a global distribution that includes the U.S.A., several European countries, South Africa, Indonesia, China, Taiwan, and most recently Japan (Waltzek and Hedrick, 2004, Tu *et al.*, 2004, Sano *et al.*, 2004). In this short review we discuss the original isolation of the virus, the important effects of temperature on the agent, some initial and more recent characterization of the virus and close with a summary of currently used diagnostic methods.

Signs of the disease

The external signs seen in sick fish include swollen and necrotic gill filaments, excessive mucus production or discolored patches on the skin (Fig. 1), and enophthalmos (Walster, 1999, Hedrick *et al.*, 2000). Internally, the kidney and spleen may be enlarged.

Virus isolation

The development of the koi fin (KF-1) cell line in our laboratory in 1997 made possible the isolation of KHV as most common cell lines used by fish virology laboratories were not susceptible to the new virus. The virus was isolated from numerous tissues of fish with signs of the disease, including the gill, kidney, spleen, gut and liver. The virus induced cell fusion and intense cytoplasmic vacuolation in KF-1 cells within 5 days after inoculation at 20°C (Fig. 2). More complete CPE or cell lysis was evident at 7 – 10 days and progressed to involve all the cells after 14 days.



Fig. 1. Juvenile koi with koi herpesvirus disease (KHVD). (A) White patches on the lateral body surface of all three fish are evident (arrows) and (B) bilateral enophthalmos (sunken eyes) in the fish on the left with KHVD compared to a healthy fish on the right and (C) the discoloration and necrosis of gill filaments.

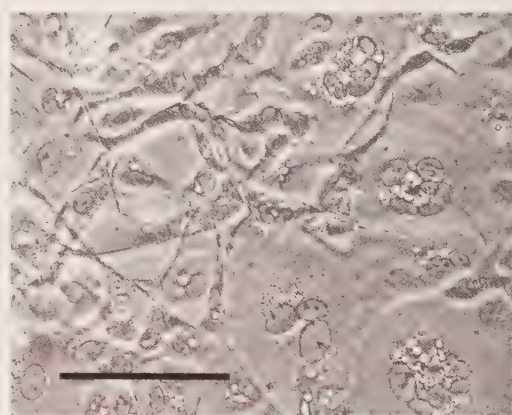


Fig. 2. Koi fin (KF-1) cells 7 days following infection with the koi herpesvirus (KHV) at 20°C.

Large and frequent cytoplasmic vacuolation is present in several cells most of which are involved in syncytium formation.

Bar = 100µm

Effects of temperature

Temperature is a dominant environmental factor that affects the onset and severity of KHV outbreaks. Most outbreaks occur during the spring and autumn (in the Northern Hemisphere) at water temperatures from 18 – 26 °C (Bretzinger *et al.*, 1999, Walster *et al.*, 1999, Hedrick *et al.*, 2000, Perelberg *et al.*, 2003). At lower water temperatures the virus can infect fish without inducing clinical signs of disease but when permissive water temperatures are again experienced the fish undergo typical disease and mortality (Bretzinger *et al.*, 1999, Gilad *et al.*, 2003, Gilad *et al.*, 2004). KHV replicates best in the koi fin cell line (KF-1) at 20 – 25°C but no growth is observed at 30°C or 4°C and only minimal growth occurs at 10°C (Gilad *et al.*, 2003). The known temperature range of KHV has led to experimental means to control the disease which include infecting fish at permissive temperatures and then shifting them to restrictive temperatures to avoid clinical disease and to induce immunity to re-infection. It appears that shifting temperatures either above or below the limits (e.g. 30°C or 13°C) will arrest development of clinical signs (Ronen *et al.*, 2003, Gilad *et al.*, 2004). Regimens of exposure and shifting to high water temperatures have been used in large scale trials in Israel to produce fish that have been called “naturally resistant” to KHV. Concerns remain whether these fish, while resistant to re-infection, may be potential carriers of the virus. Gilad *et al.* (2004) found that fish infected at 13°C did not experience clinical signs but did harbor viral DNA as detected by TaqMan PCR. Upon shifting to water temperatures of 23°C at 30 days post virus infection, the fish underwent disease and mortality. Curiously, fish held for 64 days post infection at 13°C and shifted to 23°C did not experience disease or mortality but whether these fish are immune to re-infection was not tested.

Virion characteristics

Electron microscopy of the virus present in and released from infected KF-1 cells shows a morphology and size consistent with viruses in the family *Herpesviridae*. Virions are composed of an inner capsid with icosadeltahedron symmetry of approximately 100 - 110 nm in diameter (Fig. 3).

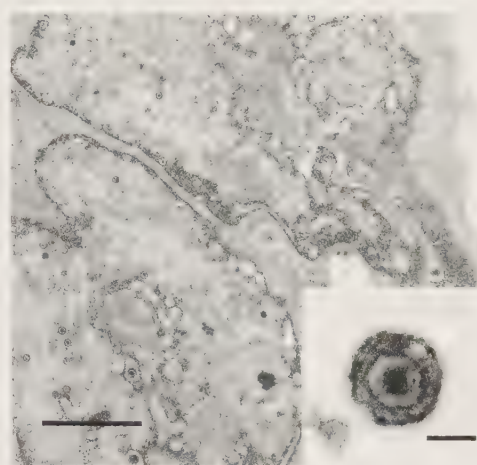


Fig. 3. Virions of the koi herpesvirus (KHV) as present in infected KF-1 cells. The effects of the virus include thickening and distortion of the nuclear membrane. Nucleocapsids of KHV are evident throughout the nucleoplasm. Bar = 500 nm. Inset figure is a complete KHV virion showing the viral envelope, the tegument (lying between the envelope and the nucleocapsid), and the hexagonal nucleocapsid. Bar = 100 nm.

KHV virions possess a tegument lying between the loosely applied envelope and the nucleocapsid that give the mature virion an overall diameter of 170 – 230 nm. Identical virus particles were observed in tissues from koi involved in epidemics in Israel and the USA. Based on the morphology and size of the virus and the sequential development in the host cell nucleus, the new virus was initially designated KHV for koi herpesvirus (Hedrick *et al.*, 2000). The same agent was isolated subsequently from koi and common carp by Ronen *et al.* (2003) although they preferred the designation of carp nephritis and gill necrosis virus (CNGV) for the agent.

Koch's postulates

The ability to isolate and then grow KHV in the KF-1 cell line provided the first steps in confirming the virus as the cause of the new disease. The virus was first isolated from fish showing signs of the new disease and the virus reproduced the disease in experimentally-infected fish (Hedrick *et al.*, 2000). Re-isolation of the virus from experimentally-infected fish confirmed that KHV was indeed the cause of this new disease. Similar studies for the virus designated CNGV were later described by Perelberg *et al.* (2003) and several other groups have also confirmed the virulent nature of KHV as isolated from infected fish.

KHV Differs from other cyprinid herpesviruses

That the new virus differed from *Cyprinid herpesvirus 1* (CyHV-1) or the agent known to cause carp pox (Sano *et al.*, 1985a, b, 1993b) was suggested by differences in the diseases caused by both agents. Additionally, anti-CyHV-1 antibodies failed to react with KHV in immunofluorescence assays (Hedrick *et al.*, 2000). Subsequently, differences in the virion proteins and genomic sequences provided additional evidence that the two viruses were distinct agents (Gilad *et al.*, 2002). CyHV-1 can cause mortality among koi and common carp but only among fish younger than 2 month in age (Sano *et al.*, 1985b). Also, survivors of CyHV-1 infections develop characteristic papillomatous-like growths commonly known as carp pox (Schubert, 1966). One additional herpes-like virus, *Cyprinid herpesvirus 2* (CyHV-2) originally named goldfish hematopoietic necrosis virus (GFHNV) has been observed by electron microscopy and has been isolated from goldfish (*Carassius auratus*) with severe hematopoietic necrosis (Jung and Miyazaki, 1995). The virus is quite difficult to grow in established cell lines (Groff *et al.*, 1998, H. Fukuda, pers. comm., unpublished

observations). Recent comparisons of the genomic DNA and virion polypeptides of KHV to CyHV-1 indicate sufficient relationships to consider them related but clearly distinct agents and that has allowed the unique detection of each virus by newly developed PCR assays (Gilad *et al.*, 2002, Gray *et al.*, 2002, H. Fukuda, pers. comm., unpublished data). The biological properties of the three cyprinid herpes-like viruses (CyHV-1, CyHV-2 and KHV) also supports the unique nature of the three agents. KHV, unlike CyHV-1, is highly virulent and induces mortality among all sizes of koi and common carp (Bretzinger *et al.*, 1999, Hedrick *et al.*, 2000, Perelberg *et al.*, 2003). CyHV-1 is lethal only for koi and common carp less than 2 mo of age (Sano *et al.*, 1985a, b). There is no evidence of papilloma formation in fish surviving KHV infection. Host range is also a clear factor separating the three cyprinid herpes-like viruses. Goldfish are highly susceptible to CyHV-2 but resistant to KHV and CyHV-2 fails to induce disease in koi injected with the virus (Jung and Miyazaki, 1995, Bretzinger *et al.*, 1999, Ronen *et al.*, 2003, Perelberg *et al.*, 2003, unpublished results).

Relationships with other herpes-like viruses from fish

The large size of the genome of KHV, which is estimated at 277 kbp, exceeds that of 250 kbp known for members of the family *Herpesviridae* (Ronen *et al.*, 2003). While genome size is not currently considered a criterion for placement or exclusion of viruses in the family, the differences in sequences so far obtained for KHV have shown little similarities to those known from herpesviruses from birds or mammals. That KHV differs but is closely related to two cyprinid herpesviruses currently classified in the family *Herpesviridae* has recently been established (Waltzek *et al.*, in preparation). Proposals are developing that involve considerable taxonomic changes to the family

Herpesviridae including the creation of higher taxa that would accommodate the large differences seen between herpes-like viruses from fish, amphibians and mollusks from those herpesviruses from birds, reptiles and mammals (A. Davison, pers. comm.). These revisions would formally place the herpes-like viruses from fish into a unique taxon within the family *Herpesviridae*. KHV would then be designated as the third cyprinid herpesvirus, *Cyprinid herpesvirus 3* or CyHV-3 (Waltzek *et al.*, in press). Formal proposals to the International Committee on the Taxonomy of Viruses (ICTV) to establish this nomenclature for KHV or CNGV are in progress.

In support of this taxonomic proposal is data from our initial studies on virion polypeptides and RFLP analyses of the genomes of CyHV-1 and KHV that clearly showed they are related viruses (Gilad *et al.*, 2002). More recently comparisons by Waltzek *et al.* (in press) of the sequences of 3 genes common to CyHV-1, CyHV-2 and KHV show a strong relationship (up to 80% homology at the nucleotide and amino acid level). This relationship provides sufficient justification to designate KHV as a third herpes-like virus from cyprinids. Additional comparisons in our laboratory of the DNA polymerase gene of these 3 cyprinid herpes-like viruses to a range of herpes-like viruses found in eel, sturgeon, trout, catfish, walleye and frogs indicate they form a group of unique viruses quite distant from other members of the family *Herpesviridae* and other viruses in general (unpublished data). Although, this group of herpes-like viruses from fish and amphibians differs significantly from other members of the family *Herpesviridae*, changes to the family mentioned above will accommodate these distantly related groups.

Current detection methods

A presumptive diagnosis of KHV disease includes observation of high mortality despite treatments for bacterial and or external parasites among koi or common carp with characteristic external pathological signs at water temperatures ranging from 18 – 26°C. Confirmatory diagnoses require demonstrations of the presence of virus by isolation using KF-1 or other KHV susceptible cells followed by polymerase chain reaction (PCR) testing of virus isolates or fish tissues directly by one of two PCR assays (Gilad *et al.*, 2002, Gray *et al.*, 2002). Unfortunately, it is often difficult to isolate or to enumerate concentrations of KHV from infected koi using cell culture methods, particularly if the fish have been dead for several hours or have been frozen. Detection may therefore, rely solely on the PCR results obtained from fish tissues.

The principal target tissues for KHV based on microscopic pathological exams and recent quantitative PCR assays of naturally and experimentally-infected koi are the gill, kidney and spleen but other tissues including the skin, brain, gut and liver, are also involved (Hedrick *et al.*, 2000, Gray *et al.*, 2002, Gilad *et al.*, 2003, Gilad *et al.*, 2004). Current diagnostic approaches most often use portions of the gill, kidney and spleen for virus isolation and or PCR analysis. Standard PCR approaches have not demonstrated the ability to routinely detect evidence for the virus in the tissues of suspected carriers of KHV, which is viewed as important to controlling the spread of the virus.

Since the initial isolation of KHV in 1998, a number of new and improved methods have been developed to detect the virus. That isolation of the virus was difficult was shown early and thus other detection methods have been rigorously pursued. The PCR method has proven to be an effective means to detect viral DNA in a number of fish tissues during the acute disease and for a period following

recovery. As many as 7 different PCR tests for KHV are currently in use in different laboratories throughout the world (Gilad *et al.*, 2002, Gray *et al.*, 2002, H. Bercovier, K. Way, M. El-Matbouli, pers. comm.). These tests have greatly improved the ability to detect evidence for the virus but leave unresolved whether carriers with persistent or latent infections occur following acute disease. Under additional development are sensitive and specific enzyme linked immunosorbent assays (ELISA). One such ELISA was described by Ronen *et al.* (2003) and it effectively detected progressive increases in anti-KHV antibodies in naturally exposed koi. A similar assay developed in our laboratory using a monoclonal antibody to carp immunoglobulin is now under field testing and preliminary evidence suggests it is quite effective in detecting prior exposures to the virus, even up to 1 year past the last evidence of clinical signs in the koi population (Adkison *et al.*, in preparation). Lastly, that the serum antibodies detected by the ELISA are at least partially protective to virulent virus challenge was demonstrated in passive immunity trials with juvenile koi (unpublished data). These studies suggest that anti-KHV antibodies may serve as useful diagnostic indicators of prior exposure to the virus and perhaps as good indicators of the immune status following virus exposure or vaccination (Ronen *et al.*, 2003).

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Prevention of a Mortal Disease of Carps Induced by the Carp Interstitial Nephritis and Gill Necrosis Virus (CNGV) in Israel

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Massive mortality of Koi and Common carp - *Cyprinus carpio* species - was observed in many farms throughout Israel, resulting in severe financial losses.

This lethal disease is highly contagious and extremely virulent, but morbidity and mortality are restricted to Koi and Common carp populations (Fig. 1).

We isolated a carp nephritis and Gill necrosis virus (CNGV), which is the etiologic agent of this disease. The virus propagates and induces severe cytopathic effects five days post infection in fresh Koi fin cell cultures (KFC). The virus harvested from KFC cultures induced the same disease with mortality of 75-95% upon inoculation of naive Koi and Common carp (Pikarsky *et al.*, 2004).

Electron microscopy revealed viral cores with icosahedron morphology of 100-110 nm resembling the herpes virus. Electron micrographs of purified pelleted CNGV sections, together with sensitivity to ether and Triton x 100 suggest that it is an enveloped virus. However, the genome of the isolated virus is a double-stranded DNA molecule of 250-300 Kbp, larger than that of known *Herpesviridae* members. The viral DNA seems highly divergent and bears only small (16-45 bp) fragments similar to the genomes of several DNA viruses. We suggest, therefore, that the etiologic agent of this disease may represent as yet unclassified virus species endemic to cyprinids (Hutoran *et al.*, 2004).

Carps, exposed to the virus at 23 °C for 3-5 days and then transferred to the non-permissive temperature of 30 °C, became resistance to a challenged infection and their



Fig. 1. The spread of the carp viral disease in Israel between 1998 and 2000.

- Area of infected farms in 1998
- Area of infected farms in 1999
- Area of infected farms in 2000

sera demonstrated a high level of virus specific antibodies (Fig. 2).

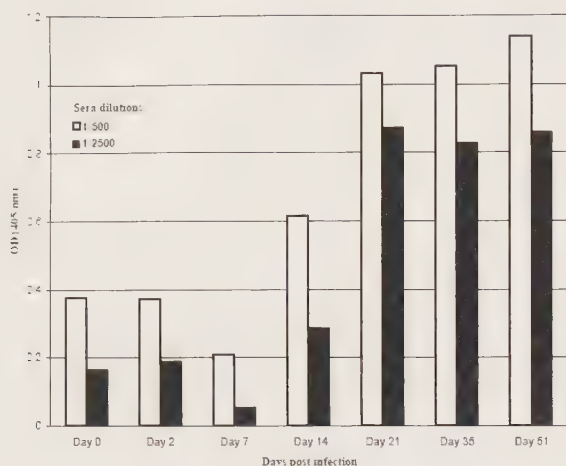


Fig. 2. Induction of anti-CNGV antibodies in carp sera following infection by cohabitation with sick fish.

We have isolated attenuated non-pathogenic viruses that render virus-vaccinated carps resistant to the disease (Fig. 3) (Ronen *et al.*, 2003). Furthermore, vaccinated fish developed high levels of antibodies against the virus. We suggest, therefore, that this attenuated virus could be used as a live vaccine for the eradication of the mortal disease afflicting common and ornamental carp fisheries in many countries (Perelberg *et al.*, 2005).

We examined the pathobiology of this disease in carp using immuno histochemistry. We found large amounts of the virus in the kidneys of sick fish, and lesser amounts in liver and brain. A rapid increase in the viral load in the kidneys was detected using both immuno-fluorescence and semi-quantitative PCR. Histological analyses of fish at various times after infection revealed signs of interstitial nephritis as early as 2 days post-infection, which increased in severity up to 10 days post-infection. There was severe gill disease evidenced by loss of villi with

accompanying inflammation. Minimal focal inflammation was noted in livers and brains.

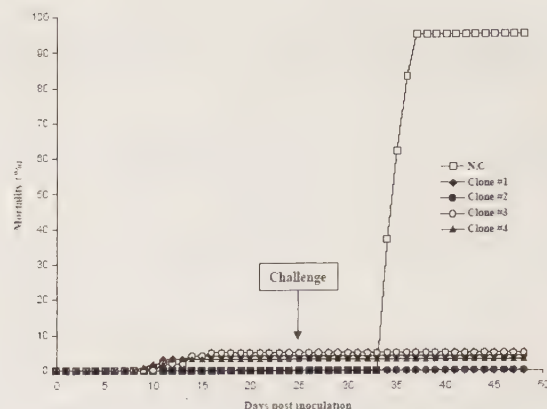


Fig. 3. Mortality rate in vaccinated fish following a challenge infection.

Two diagnostic methods for identifying the CNGV in live fish are in use in our laboratory: Using PCR with authentic primers is applied on blood, kidney or gills DNA samples and immunological diagnostic kit. The immunological kit is designed to be a simple, rapid and low cost diagnostic kit, which will be appropriate for retailers as well as hobbyists to identify the CNGV in presymptomatic fish. We believe that these means will be instrumental in preventing the distribution of sick fish world wide.

Conclusion

- 1) The causative agent of the disease in common carp and koi population is an enveloped DNA virus with a core of 110 nm with an electron dense region.
- 2) We have named the virus Carp Nephritis and Gill necrosis Virus (CNGV) according to its pathological manifestations.
- 3) There are some morphological similarities between CNGV and herpes viruses.

- 4) The CNGV genome is composed of a ca~ 277Kbp DNA molecule, larger than the other *herpesviridae* members.
- 5) The viral DNA sequences resolved so far suggest that the viral genome is highly divergent from that of *herpesviridae* members, and is not similar to other DNA viruses.
- 6) We have selected an attenuated viral strain following multiple transfers in tissue culture. The attenuated virus is non-pathogenic, induces humoral response in vaccinated fish and renders them protected against challenge infections with the wild type virus.

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KHVD, Diagnosis, Control, Research and Future in The Netherlands and Europe

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In recent years koi herpesvirus disease (KHVD) has quickly spread over Europe. In several European countries the first KHVD cases were observed in 2002 (Italy, Denmark) and 2003 (France, Austria, Switzerland). In contrast to Western Europe, where koi and carp are mainly kept as ornamental fish, in Eastern Europe carp is farmed for consumption and KHVD could have a huge impact on the carp industry. At the moment however, Eastern Europe still seems free from KHVD. However, common carp exported from Poland to Germany were shown to be KHV positive. The highest incidence of KHVD cases in Europe are found in Germany, England and the Netherlands.

Germany has had over 250 cases since 2002. England had 36 cases in 2002, and more cases followed in 2003.

In the Netherlands a steady increase was seen from 2 KHV positive cases in 2001 up till 27 cases in 2003 (Table 1).

Concern for these countries is that KHVD could possibly not only be restricted to koi-

trading and private ponds but already be spread into the natural environment.

At the moment however there is still no evidence for this. As KHVD is not notifiable for the European Union (EU), the control on spread of the disease within the EU is minimal.

The majority of the KHV cases in the Netherlands were detected by PCR (Gilad *et al.*, 2002), with or without using gill histopathology as a confirmatory method (Fig. 2). In a few cases the herpes virus could be cultured successfully on Epithelioma Papilloma Carposum (EPC) or Koi Fin-1 (KF-1) cells (Fig. 1).

In 2001 we succeeded in isolating the virus for the first time in the Netherlands from a clinical diseased koi. The inoculated sample of gills and pooled internal organs showed cytopathic effect (CPE) at the 2nd passage on EPC cells

Table 1. KHV in The Netherlands.

Year	KHV cases	Host
2001	2 positive cases out of 6 (PCR): 1 virus isolation	Only koi affected. Up till now no cases in carp
2002	6 positive cases out of 30 (PCR): 1 virus isolation	
2003	27 positive cases out of 63 (PCR/hist.)	



Fig. 1. Virus isolation of KHV on KF-1 cells. Left, control KF-1 cells. Right, 8 days post infection at 20°C. Confirmation of KHV with EM and PCR.

Unfortunately the sensitivity of viral isolation is inadequate for diagnostics of KHV. Additional methods, as PCR, are required for correct diagnosis of the disease.

Currently effort is put into developing alternative detection methods as immunofluorescence tests (IFT) to support diagnostics of KHVD (Fig. 2).

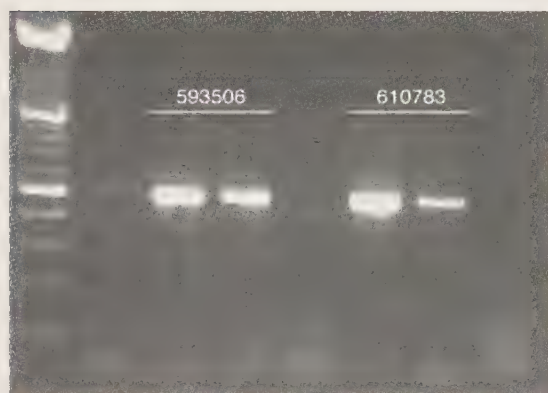


Fig. 2. Example of detection of KHV with PCR method according to Gilad *et al.* 2002. Two samples, for each sample gill tissue (left) and pooled internal organs (right). DNA isolated from gills and internal organs with commercial QIAgen kit.

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Indonesian Experience on the Outbreak of Koi Herpesvirus in Koi and Carp (*Cyprinus carpio*)

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Abstract

Koi Herpes Virus (KHV) is a new emerging disease known to cause gill and skin damage in koi and carp (*Cyprinus carpio*). The disease suspected to have been introduced into Indonesia through importation of koi from Hongkong. It is currently occurring in Indonesia since March 2002 starting in the area of Blitar in East Java. Since then it has been spreading rapidly throughout Java Island, Bali, southern part of Sumatra, East Kalimantan and Central Sulawesi. The disease cause very high mortality (80-95%) to both koi and common carp with estimated losses of more than 150 billion rupiahs (equal to US\$15 M) as of December 2003. To prevent the spread of the outbreaks to other islands, the Government of Indonesia issued Ministerial Degrees that declared Java and Bali Islands as an isolated area of the disease and moving koi and carp from Java and Bali Islands to other islands are strictly prohibited or should follow quarantine check for KHV. In addition, importing koi and common carp is permitted only from free KHV countries. A Task Force consisted of international, national and local experts were organized to conduct emergency assessment of the disease situation through epidemiological investigations, field observations and laboratory examinations. Information on KHV was disseminated widely across the country with the use of TV, radio, newspaper, posters, pamphlets and technical guideline. In farm level, biosecurity concept was applied to reduce the risk of KHV outbreak. The implementations of government regulations pertaining the outbreak and key components of biosecurity are discussed in this paper.

Key words: Koi herpesvirus (KHV), Outbreak, Control, Indonesia

Introduction

Koi carp (*Cyprinus carpio*) is an economically important freshwater ornamental fish cultured in Indonesia, while common carp (*Cyprinus carpio*) is the main freshwater fish species in the country. Annual production of cultured common carp in last five was as follows: 56,546 MT (1998), 57,278 MT (1999), 75,322 MT (2000), 76,475 MT (2001) and 83,885 MT (2002) (DGA, 2003). Fifty percent of this annual production is contributed by West Java. There are three type of common carp culture systems, i.e. floating netcages culture in the lake or

reservoir, running water (raceway) in the river or stream and earthen ponds. In addition to the cultured common carp, the fish are also found in wild habitats such as rivers, lakes and reservoirs.

Koi herpesvirus (KHV) is a new emerging disease known to cause gill and skin damage in koi and carp (*Cyprinus carpio*). The disease was first recognized in Israel and USA (Hedrick *et al.*, 2000). Since then it has been reported in Europe including Germany (Hoffman *et al.*, 2002) and United Kingdom (Way *et al.*, 2002). In Asia, KHV has been reported in Indonesia (Sunarto *et al.*,

2002), Japan (Sano *et al.*, 2004) and Taiwan (Tu *et al.*, 2004).

This paper describes the status of KHV infection in Indonesia and the experience of the country dealing with this disease outbreak. The implementation of the government regulations pertaining prevention of KHV outbreak and key components of managing the disease in farm level through biosecurity concept are also discussed in this paper.

History

The history of KHV in Indonesia including the first 3-episodes of the outbreak was chronologically described by Sunarto *et al.* (2002). The first episode of mass mortalities of cultured koi (*Cyprinus carpio*) was recorded in March 2002 in Blitar, East Java. It occurred after heavy rains among new fishes introduced from Surabaya, the capital city of East Java. The fish were imported from China through Hongkong in December 2001 and January 2002. Blitar is well known as the centre for koi production in the country. The koi including the infected one were distributed over the country, with to Central Java, West Java and Jakarta as the main market.

The second disease outbreak occurred in cultured common carp (*Cyprinus carpio*) during the end of April 2002 in Subang regency, West Java. The gross signs of the diseased common carp were extremely similar with that observed previously in koi carp.

Subang is one of the production centres of common carp in West Java. Since then the outbreaks spread to neighbouring regencies mainly through fish movements.

The third episode of the outbreak occurred in end of May to early of June 2002 in cultured common carp in floating netcage at Cirata Reservoir, West Java. Weeks before the outbreak, farmers introduced common carp from Subang region due to the low price of fish.

The fourth episode of the outbreak occurred in February 2003. The outbreak affected cultured common carp in Lubuk Lingau regency, South Sumatera. The gross signs of the diseased common carp were extremely similar with that observed previously in koi and carp in Java islands. Common carp farms at Lubuk Lingau were infected with the disease coming from Cirata Reservoir, West Java through fish transfer by traders. The outbreak then spread to neighbouring district and province including Bengkulu in the south and Jambi in the west.

Geographical Distribution

A survey of KHV was conducted in April 2003 as part of FAO's Technical Cooperation Project entitled Health Management in Freshwater Aquaculture (Cameron, 2003; Rukyani, 2003). The main objectives of the survey were to determine the extent and the distribution of the population at risk against KHV, the current distribution of the disease and extent of the disease.

In order to achieve these objectives and meet the criteria listed above, a rapid survey at district (*kabupaten*) level as a unit of interest was conducted. The survey was at all districts in the country (353 districts). A printed questionnaire, which is submitted to the district fisheries services, was used to collect data. The questionnaire was pre-printed with the name of each district and province, as well as the district code, to simplify the data entry.

The results showed that KHV has infected most of district in Java islands, Bali, southern part of Sumatera, East Kalimantan and Central Sulawesi. The geographic distribution of KHV in Indonesia as of February 2003 was illustrated in Fig. 1.



Fig 1. Geographical distribution of KHV in Indonesia. Red colour indicated KHV infected areas.

Epizootiology

Host. Morbidity and mortality due to the virus were restricted to koi and common carp (*Cyprinus carpio*) populations.

Carrier. It is not known yet whether any fish species other than *Cyprinus carpio* harbour the virus and act as carriers of KHV.

Mortality. The disease cause very high mortality (80-95%) to both koi and common carp.

Mode of spread and transmission. The disease transmits from diseased fish to healthy fish occur through direct contact, infected tissues or dead fish and contaminated water and equipments. Local spread of the disease occurs through contaminated water source and irresponsible movement of infected fish. Long distance spread of the disease mainly occurs through irresponsible movement of infected common carp and unintentional spread through koi show.

Clinical sign. The infected fish were lethargic, showed loss of balance and gasped for air. Common gross sign including sloughing off the epithelium with loss of mucus and rough appearance of the skin or showed a blister-like lesion on the skin, haemorrhages of operculum, fins, tail and abdomen, and severe gill damages (Fig. 2).



Fig 2. Clinical signs of KHV-infected carp. Note the gills damage.

Environmental factors. Most of the outbreaks occurred when infected fish introduced to non-infected fish/farm/ area. The heavy rain prior to the initial outbreak made us to consider the possibility of an environmental triggering factor, such as water temperature. Another environmental factor that may trigger the outbreak is low water quality. However, research was needed to elucidate the environmental and other factors that trigger the outbreak.

Diagnostic Technique

Diagnostic methods against KHV was established based on standard of FAO/NACA/OIE (Bondad-Reantaso *et al*, 2001) i.e. environmental observation and clinical sign (diagnostic level 1), histopathology changes (level 2) and molecular biology (level 3). In order to minimise misdiagnosis of KHV in farm level (diagnostic level 1), a 'case definition' was established, i.e. high mortality of koi or common carp (*Cyprinus carpio*) within 7 days, in which the fish shows gill damage, with or without any other clinical signs.

Diseased fishes showed consistent histopathological findings including

intranuclear amphophilic inclusion bodies with peripheral chromatin margination were observed within the gill's epithelium. Similar inclusions were also observed within the kidney tubular epithelium (diagnostic level 2).

PCR detection (diagnostic level 3) of KHV was carried out using specific primers set developed by Gray *et al.* (2002) and Gilad *et al.* (2002).

However, since histopathological changes due to KHV infection were not obviously observed in most of the diseased fish, histopathological changes was hardly used for diagnostic (level 2). Currently, the diagnosis of KHV in Indonesia was based on 'case definition' (diagnostic level 1) and PCR detection (level 3).

Public Health

The fact that some of the diseased fish showed blister-like lesion and the word of 'herpes', which is associated with herpesvirus in human, has increased concern of consequences of consuming diseased fish. There is however, no scientific evidence that KHV is a zoonotic disease.

Socio-Economics Impact

The first report regarding the economic losses due to the outbreak was made by the head of the Association of Ornamental Fish Culture of Blitar regency, East Java. They reported that in Blitar alone, the outbreak destroyed high quality koi carp belong to 5,000 fish farmers with economic losses more than Rp. 5 billions (US\$ 0.5 millions) within the first 3 months period of the outbreak. As of July 2002, the NACA's Task Force estimated that the loss revenue of the sector and the socio-economic impact to the rural farming communities was in the region of US\$ 5 millions.

As the outbreaks continued and spreading to new areas, the socio-economic impact due to the outbreaks was escalated. Directorate of

Fish Health and environment (DFHE) estimated that as of December 2002 and 2003, losses due to KHV were US\$10 million and US\$15 million, respectively.

Control Measures

There are no known treatments for fish infected with KHV, however, a number of preventive measures have been taken by the Government of Indonesia to reduce the spread of the disease in national level.

Soon after the first outbreak in March 2002, the Directorate General of Aquaculture (DGA) issued circulation letter pertaining the occurrence of the outbreak and prohibition of movement of infected fish from infected area to non-infected area. With regard to KHV outbreak, 2 Ministerial Decrees have been issued. Ministerial Decree No. 28, June 2002 followed by Ministerial Decree No. 40, October 2002 were issued in order to protect the country from the introduction of the disease, to prevent wider spread of KHV and to minimise economic losses on koi and common carp culture. The Degrees declared Java and Bali Islands as an isolated area of the disease and moving koi and carp from Java and Bali Islands to other islands are strictly prohibited or should follow quarantine check for KHV. In addition, importing koi and common carp is permitted only from free KHV countries.

However, the circumstances make the application of the Decree No. 40/2002 less effective, because trucks with live fish often cross the strait without reporting to the quarantine checkpoint office. The application of Decrees is relatively ineffective as the disease leak to South Sumatra in February 2003. The spread of KHV disease to the whole of Sumatra is just a matter of time unless there is other policy to stop the infected live fish movement within Sumatra islands. Therefore, the Governor or provincial authorities of uninfected regions should be encouraged to regulate the

transportation of live carp from Java to their provinces (Prayitno, 2002).

Besides issuing rule and regulation, the government had also quick response to the outbreak including organizing local disease task force, reporting the outbreak to the OIE (Rukyani, 2002) and seeking assistance from international agencies. The last has lead to the formation of task force from Network of Aquaculture Centre in Asia-Pacific (NACA's Task Force) and project from a Technical Cooperation Programme from Food and Agriculture Organisation (FAO-TCP project).

The NACA's Task Force consisted of international, national and local experts were organized to conduct emergency assessment of the disease situation through epidemiological investigations, field observations and laboratory examinations. Information on KHV was disseminated widely across the country with the use of TV, radios, newspapers, posters, pamphlets and technical guidelines.

Under FAO-TCP projects, various issue regarding policy and legislation on fish health management in Indonesia were addressed. These are include enhance national regulatory framework and development of national strategy on fish health management. Other related issues on fish health management such as strengthen diagnostic and control capability, improve surveillance, monitoring and disease reporting system, human resource development and public awareness are being addressed. A web-based information system on disease fish health management (www.ausvet.com.au/indofish) has been constructed through the project.

Soon after the outbreak, the government provides aids to affected carp farmers. These include broodstocks, disinfectant, training, dissemination of information and consultancies. A website (www.ditkeskanling.go.id) was also established to provide information on fish health management including KHV outbreak.

As there are no treatments for KHV, attempt to control the outbreak in farm level is focused on the prevention of getting infected with the virus and subsequently reducing the mortality once the farm is infected with the disease.

Biosecurity. Most outbreaks occurred after the introduction of new fish from infected area or getting infected through contaminated equipment and water source. Therefore, biosecurity concept may be applied to prevent or reduce the possible KHV infection. For non-infected farm, it is highly recommended to use only KHV-free seed and broodstock and to avoid introduction of new fish, particularly from infected area. If introduction of new fish is unavoidable, it is recommended that all new fish be quarantined for at least 2 weeks, together with sentinel fish at permissive water temperature between 18-28°C.

For farm/area where KHV is presently endemic, eradication of KHV from the farm should be done prior stocking with KHV-free stocks. It should include removal of all fish (particularly all susceptible species) from ponds, reservoirs, and water canal prior to restocking, drying out and liming the ponds and disinfections of contaminated equipment.

Once the virus has been eradicated from a site, it is important to prevent its re-introduction. It is likely that infection is spread by affected fish, as well as by contaminated water and equipment. Accordingly, the following measures should be considered: seed and broodstock should be obtained from KHV-free area, all wild carps must be rigorously excluded from farms in endemic areas, water should be obtained only from an KHV-free sources, and equipment which may have been used at infected sites must be disinfected. However, these practices may only be effective for isolated farm such as koi farm, but not for cage culture in the lake/river and raceway culture in the river.

Good management practices. Good management practices (GMPs) should be applied in koi and carp culture. It seems that GMPs could decrease the mortality due to KHV infection. It is recommended to use only KHV-free seed and reduce stocking density. There are scattered reports that transferring diseased fish from raceway to earthen pond will decrease the mortality and prevent further losses. This practices reduced the stocking density and stable water temperature, hence provide a better environment to the diseased fish. The use of immunostimulant and vitamin C may increase non-specific immunity of fish against KHV.

It is recommended to remove dead and moribund fish from the pond, as they are a good reservoir for the virus. In order to reduce the mortality, curative measures were also applied to treat secondary infection of parasite, fungi and bacteria. Enrofloxacin (5-10 ppm) were used to treat secondary bacterial infection. Dyvon (Dichlorophos) of 1-5 ppm and Benzalkonium chloride (BKC) of one drop/100 L were used to treat Argulus and other parasite infection.

Despite of the difficulties on the control of the outbreak in common water body such as in the raceway and netcages in a reservoir/lake, GMPs and biosecurity concept seems to provide an alternative control strategy in farm level. However, further research need to be done to identify other key component of biosecurity and GMPs, which effective to control KHV outbreak.

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The Status of Viral Diseases of Carp in Korea: Its Control and Research Development

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The carp (*Cyprinus carpio*) is the most common species of cultured fish, in particular, common carp and Israel carp have been cultured as an edible fish for several decades in Korea. Viral diseases of carp have never been reported until 1998 despite its long history of aquaculture in Korea. However, mass mortality occurred in populations of cultured carp from 1998 to 1999 resulting in a drastic reduction of carp production in Korea. The infected fish showed dark coloration, excessive mucus secretion from the surface and gills and branchial necrosis (Figs. 1 and 2).



Fig. 2. Internal characteristics of VSNC: Branchial necrosis and white patch on gill.

In moribund carp, the clinical signs were formation of white patches on body surface or gills due to excessive mucus secretion. Dermal lesions caused by secondary infection were also found.

So, we called that the viral disease is a “viral systemic necrosis of carp (VSNC)” based on their clinical signs. The mortality was the highest at 20~28°C, and there being virtually no occurrence at below 20°C or above 30°C. Although the characteristics of VSNC are similar to those of KHV, PCR tests proved that it was not KHV.

Oh *et al.* (2001) reported that the virions with 70~80nm diameter were presumed to be a causative virus in the kidney and spleen tissues of infected fish. They also reported that the artificial infection using a viral

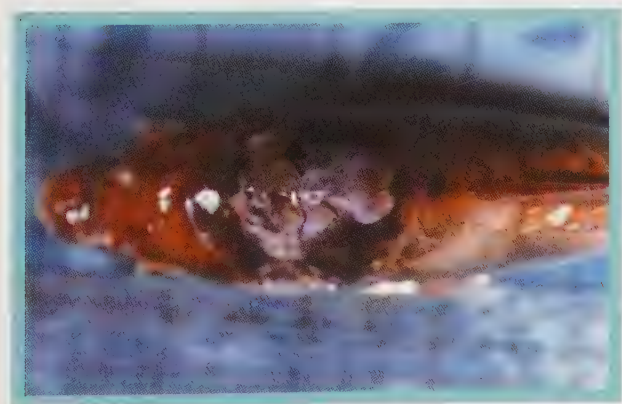


Fig. 1. External signs of moribund fish show the characteristic of white patches.

Several cases were reported that dermal ulcers were noticed due to secondary infection by bacteria or parasites.

suspension of 10^3 TCID₅₀/mL obtained from the infected FHM (fathead minnow) cells, gave 80 to 100 percent mortality in carp (Fig. 3).

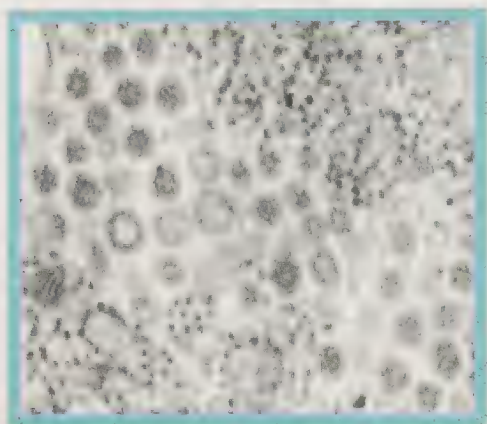


Fig. 3. Virions (Oh *et al.*, 2001).

Kim *et al.* (2002), as a result of conducting gene cloning of the virus, suggested that the virus being a new species different from the known reported viruses of carps such as grass carp reovirus (GCRV), grass carp hemorrhage virus (GCHV), koi herpesvirus (KHV) and spring viremia of carp virus (SVCV).

Unlikely such findings, Choi *et al.* (2004) found a herpes-like virus of 82nm size within the nucleus of spleen cells from infected carps (Fig. 4).

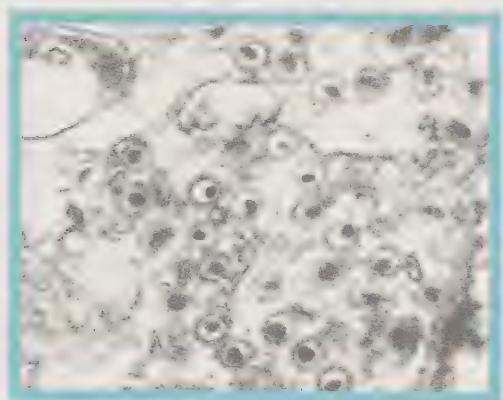


Fig. 4. Isolation of herpes-like virus from infected carp (Choi *et al.*, 2004).

During the past three years, the present study was established to development of prevention technologies for epidemics in order to protect the carps from the viral diseases.

First, two types of inactivated vaccines were prepared with the viral suspension of VSNC that obtained from the infected FHM cells: formalin-killed vaccine (FKV: 0.4% formalin) and heat-killed vaccine (HKV: 60 °C, 1hour).

The lysozyme activity of serum and chemiluminescent responses of head-kidney leucocytes showed increasing in the vaccinated fish, and as measured by ELISA (enzyme-linked immunosorbent assay), vaccinated groups showed a significant increasing in the virus-specific serum antibodies between 2 week post-first injection and 6weeks post-boost injection.

Results of the virus challenge showed that the fish vaccinated with FKV have induced protective immunity, while HKV injection hardly provided protection. And secondly, the antiviral activity of carp was examined using the potent inducers of interferons such as poly inosinic: cytidylic acid (Poly I:C) and spring viremia of carp virus (SVCV).

The results showed that carp injected Poly I:C developed an anti-viral protection which resulted in lower cumulative mortality against SVCV than untreated fish did. Moreover, in vitro study showed that head kidney leucocytes produced an interferon-like cytokine (ILC) after stimulation with Poly I:C (Fig. 5).

Since 1998, many studies have been conducted in diversified aspects to find out the causative virus of the diseases and prevention measures against viral disease of carp in Korea. However, identification of the virus was not clear and effective control measures were not established so far. Therefore, further studies on the causative virus including characterization and infection mechanisms are required.

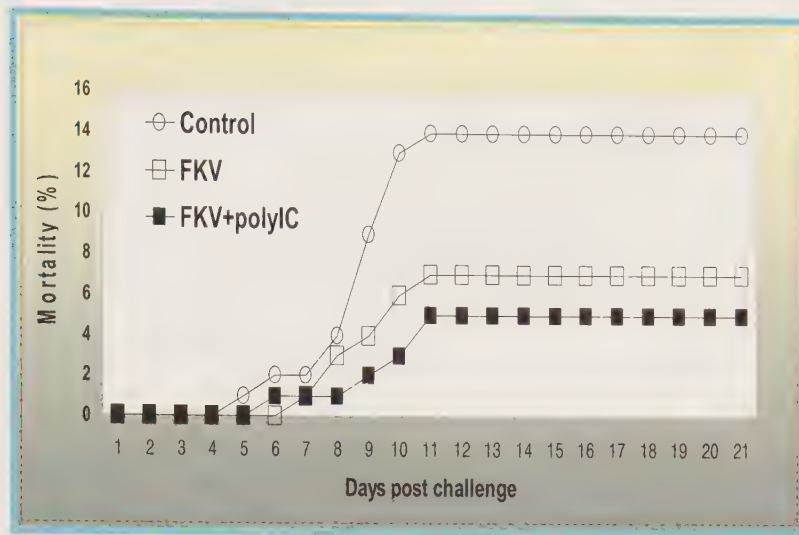


Fig. 5. Effect of Poly I:C on cumulative mortality against SVCV in vaccinated carps with formalin-killed SVCV.

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Viral Studies on Carp Disease in China --- with a Special Reference to Herpesvirus on Common Carp---

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Abstract

The present paper mainly deals with the general situation of the viral diseases affected on the Chinese carps in a recent decade in China. The viral disease is highly characterized by the fast transmission, great mortality and being difficult to control which has caused a heavy loss in the aquaculture systems. The paper further and specifically introduces the general status of herpesvirus on carps in China and preventive measures against the outbreak of the disease from the ecological point of view and the environmental issues.

Key words: viral diseases, Chinese carps, herpesvirus on carps, preventive measures

Since 1980s, there have been a rapid development of aquaculture in China, the percentage is getting higher and higher in the position agricultural development and turning one of the four major core production activities (grain, meat fish and eggs). In 1998, the fish production in China was up to 38 million tons. However, the fish diseases, particularly the virus disease becomes more and more prominent, epidemic which lead to a heavy loss in the aquaculture activities in the recent years. Due to a long time-length of incubation period, complex characters and high infection, the fish suffers from a high mortality. Also due to the small size of the pathogenic virus and the copying characters, it has a strong resistance against the antibiotics, while the chemicals application often leads to the death of the host prior to that of the pathogens. Therefore the treatment of the virus diseases is encountered with a great difficulty. The following is the general situation of the virus disease happened in China aquaculture systems.

1. Virus and morphologies

After the isolation of the first strain of the virus affected on fish, there have been reports over 70 virus with 15 families, which are popular with the most animals. In the information collected on virus studies on fish diseases in 1970s-1980s, there was also information on virus diseases with the Chinese grass carps. At the same time there were also new strains of virus discovered and isolated.

Table 1. Some important virus pathogens with fish diseases.

Name of the virus	Host	Classification	Symptoms
Channel catfish virus	Channel catfish	Herpesvirus	Acute haemorrhage
Reoviridae of G/C	Grass carp	Reoviridae	Haemorrhage
Salmo herpesvirus	Salmo & Oncorhynchus	Herpesvirus	Anaemia, bleeding
IHNV	Salmo & Oncorhynchus	Rhabdoviridae	Congestion on fins
IPNV	Salmo & Oncorhynchus	Birnaviridae	Eyes & abdominal enlarged, unbalance in swimming
Japan Iridoviruae	Eels	Iridoviruae	Anus congestion & over mucusing
Oncorhynchus masou virus	Oncorhynchus	Herpesvirus	Unbalance in swimming and abnormal respiration
Bass Iridoviruae	Bass	Iridoviruae	Unbalance in swimming
Spring haemorrhagical virus	C/C,S/C, Cr/C	Rhabdoviridae	Difficult in breathing
Esox lusius (northern pike) Rhabdoviridae	Pikes, G/C	Rhabdoviridae	Sudden death of the fish in mass
VHS virus	Salmo & Oncorhynchus	Rhabdoviridae	Internal bleeding, swollen with liver and spleen
Cod pancreas virus	Cod fish	Adenoviridae	Skin damaged and ulceration
Salmo bladder virus	Salmo	Retroviridae	Rumor causing
Nerve necrosis virus	S/C	Nodaviridae	Nerve necrosis
Siniperca infectious anaemia virus	Siniperca	Paramyxoviridae	Anaemia
Epidermis blood-producer necrosis virus	Oncorhynchus Bass, Channel catfish	Iridoviruae	Blood-producer necrosis

2. *The examine and diagnosis of fish virus diseases*

The earlier examination and diagnosis of the fish diseases will certainly reduce a loss in aquaculture process. The methods of diagnosis are varied but mainly are the following types:

2.1 Biological checks: With the characters of the host that is vulnerable to the disease infection, the host is served as an indicator for testing. One test with crucian carp which is similar to the characters of grass carp sensitive to the disease outbreak showed out immediate results by artificial infection through injection or bathing. The fish got infected in 1-2 weeks with the inoculation. Therefore crucian carp can be used as a biological indicator to induce the outbreak of the disease.

2.2 Microscopic observation: From the experience and practice, the special characterized data and indicator are summarized. The microscopic observation is necessary and useful tool in checking and diagnosing the diseases. Much reliable information are collected in this way.

2.3 Cell culture: Cell culture is basic in studying the virus. It is also a traditional way in diagnosing the diseases affected with virus. The normal practice follows the better understanding of the cell highly vulnerable to the virus.

2.4 Molecular biology: Establishing the reliable methods for checking and diagnosing the fish disease is a positive way for fish disease prevention and control. The molecular biological approaches are as marked antibodies that are used to determine the viral protein, nuclear acid probing or PCR. All these methodologies are also applicable to all the vertebrate animals.

2.5 *A few methods of virus examination:*

There are quite many new and improved methods for examining IHNV on fish virus. However, it demands much previous preparation. Cell culture is a good approach, but it is not as sensitive as desired.

3. *Prevention and control of the virus disease and preparation of the vaccination*

To the virus disease, till present there is no yet any effective drug for this treatment. The expertise have long insisted that there should not be an overused quantity of the drugs, it is considered that it will reduce the fecundity and quality of the fish spawning and reduce the disease resistance of the animal itself with a heavy residue leading to the pollution of the water and environment. Selection, cyto-engineering, genetic engineering will help breed new varieties against the diseases. The herbs application is also an effective way in controlling the disease outbreak. It is quite practical but vague in its mechanisms.

4. *Fish herpesvirus diseases*

Herpesvirus is available in the natural environment like many other pathogens, it has a specific host selection. In the past few years, success has already been achieved in finding out a few virus with herpesvirus. Some of the herpesvirus are rumor-causative, but some can cause the total body infection with a high mortality. There have been a good number of reports describing infectious status with herpesvirus on *Anguilla japonica* and *Anguilla anguilla*.

Herpesvirus on channel catfish is caused by its fingerlings in the southern part of USA, other countries from Central America that introduced the fish successfully isolated the strains of the virus. But there have been not yet any reports in Asian countries.

In Japan, there have been reports on herpesvirus with common carp. It is confirmed that herpesvirus is a rumor

causative for Cyprinidii and a pathogen of smallpox of European carps. So far there have been found 4 kinds of herpesvirus from Salmoni: NeVTA (from *O. nerka*), OMV (from *O. masou*); YIN (from *Yamame* of *O. masou*) and CSIV (from *O. kisutch*). In a rainbow trout farm in USA, the brooders died at 50% after spawning, it was found that herpesvirus is available in the egg yolk.

4.1 SVC is a secondary disease meanwhile confirmed by OIE, NACA and FAO: The fish suffers from an acute hemorrhage mainly attacking one-year old common carp fingerlings. Other fishes not Cyprinidii are also vulnerable to its outbreak. The dead, diseased fishes and pathogen-carriers are all the causes of the diseases. The fish lice can be for its transmission. The outbreak of the diseases can be only at the temperature of lower than 15°C. The mortality reaches 80-90%.

Pathogen: It is the herpesvirus with a 150nm in diameter in size, there is a capsule outside in spherical shape. It causes a change and mutation in the critical areas of the fish body. Under the conditions of 15-18°C, the fish (common carp) is infected by either scratches on injections, the superficial areas of the fish show a smallpox.

The symptoms and pathological changes:

The typical change is the skin hyperplasia. The diseased fish shows the white spots on the skins like wax called smallpox which are closely attached to the fish skins. The smallpox are getting bigger and more in number till covering the whole fish body (Fig. 1). It leads to a zero value in marketing. The epidemics are popular at the temperature of 15°C. The fish suffers and is overloaded till its death. After the dissection, the guts and kidneys are congested and heavy inflammation, the intestinal mucus membranes fell off, the intestinal content is full of the pus-like substances. The intestines are clear with inflammation with a light blue color, some with reddish color.

Diagnosis: The cell tissues are obtained from the liver, spleen, kidney, brain or sperm, eggs inoculated to the FHM or EPC cell for isolating virus (Fig. 2). The cell will meet a good mutation at the temperature of 20°C. The tests will be further conducted with neutralizing test, immune fluorescent test or PCR for final determination (Fig.3).



Fig. 1. The fish diseased with smallpox.

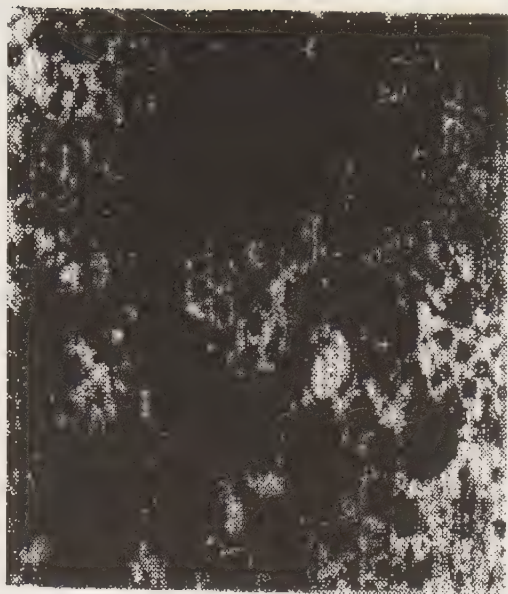


Fig. 2. Large amount of the viral granules are available in the nuclear spasm (x 24600).



Fig. 3. Smallpox: the matured viral grains are released to the superficial areas through follicles (x 13000).

4.2 Eel Virus Disease (*Eel mouth disease*)

It was firstly reported in China in 1992, it was found out in the HB cell of the eels. The virus is 20-side sphere which is composed of a core and capsule. The average diameter is about 70 nm, while the core diameter is 40 nm in size. The suspended solution with bacteria-free from the heart and spleen of the diseased fish are used to infect the healthy fish. The heart and the liver turned to gray in

color. The fish can not close the mouth after opening.

4.3 *Herpesvirus of turbot (Tarpops oligolepis)*

Characters: The virus is sphere-like with capsules the diameter is 120-220 nm. The available reports on the size of the herpesvirus (turbot) is 200-220 nm in diameter. It is transformed and copied in the host.

Pathology: Herpesvirus caused a great loss over the turbot (at 4-month old) in the farmed conditions. A large number of abnormal giant cells are found in the epidermis of the dorsal part and gill structures. The disease was also found in the farmed turbot in Demark. The fish is not active, move less, seems less oxygen. Dropsy and fallen-off of the intestines are observed. Therefore great pathological changes have taken place over the gills, skins, fish heart, guts and kidneys. Herpesviurs are clearly observed from the gill giant cells. Clinic symptoms are not very clear sometimes. Technical people further observed that turbot fingerlings from France was infected with herpesvirus which are mainly attached to the skin and the gills of the fish. The size of the virus reached over 80um, but it has a less lethal effect in infection and mortality.

5. Countermeasures against virus diseases

The outbreak and epidemics of the fish disease are closely related to the environment and the resistance of the animal itself. In the light of the practical experience gained from both research and the farmers the disease prevention and control should be focused on the improvement of the aquatic environment, removal of the pathogens and increase of the self-immunities.

5.1 Improvement of the aquaculture environment with ecological approaches

A. Water quality improvement: The harmful metabolic substances are washed and diluted with fresh water to improve the water quality. Pond preparation should be done thoroughly to eradicate the wild animals, predators and other food competitors.

B. Water temperature regulations: Most of the parasitic pathogens are only adapted to the temperature of 20°C. The reproduction of the pathogens can be blocked off by water temperature regulations, moreover, it can

accelerate the growth rate of the cultured objects.

C. Minimizing the stress: If water temperature is changed in a big range, pond silt is worsened, the feed quality gets bad, there is a sudden rain like thunderstorm leading to the change of the pH value, disturbance of netting, noise, misuse of the chemicals, there should be a heavy impact over the stress of the cultured animals resulting to the lower resistance of the diseases.

D. Sound ecological prevention and control: This is to maintain the balance between the cultured animals in the controlled conditions. The cultured ponds can be added with the PSB that can turn the harmful substances into good aspects. Predators can be used to remove the disabled, diseased.

5.2 Tight control of the pathogenic transmission.

A. Establishing vaccination system: In the process of the exotic fish introduction, careful steps are taken to prevent the pathogens along with the fish. Generally the virus disease has a long and potential period before its outbreak. The fast diagnosis process should be established to find out immediate roots of the diseases.

B. Sound environmental control: In the cultured conditions, there are large quantities of the disease-caused and non-disease-caused bacteria, while the later is the balance between the cultured environment and the animals. The large quantity of the chemical application in a great extent harms kill the disease-caused bacteria, but meanwhile it also damages and kills the non-disease-caused bacteria which are beneficial to the culturing conditions. Therefore the effective approaches to maintain the sound conditions for the cultured animals are to manipulate, monitor and manage the water quality.

C. Disinfectants: The disinfectants are categorized into two types: oxidized and non-oxidized chemicals, the former like potassium permanganate (PP). The effects are closely related to the water temperature, pH, ammoniac concentration, suspended matters, but it is poor to the virus killing, the later like copper sulphate, formalin is poor in disinfection, moreover great side-effects, it is no use in virus infection.

D. Biological control: If the polyculture is conducted, some of the cultured animals can help remove the disease pathogens for more important animals.

5.3 Micro-ecologies to control the outbreak of the diseases

A. The basic principles: When the cultured animals are under the healthy conditions, there is a dominant population of the micro-organisms with relatively stabilization. This micro-organisms can both be beneficial to the growth of the cultured animals and also restrain the harmful organisms that might cause bad effects. At the normal conditions, the following three components are closely related to the environmental equilibrium: aquatic animals and plants; micro-organisms and environment.

B. Less use of the antibiotics: Over use of the antibiotics often leads to a heavy damage of the environment, high resistance against the new chemicals and greater quantity of the chemical application. The fishery chemicals should be more directive and effectively focusing on specific diseases. Strict avoidance should be made for the general use of the chemicals that might hurt the fish kept in the same areas.

C. Application of the microbiological products: Dominant population of the microbiology is established for the farmed objects internally and externally, for example, PSB can be applied and cultured in the

controlled conditions. It is not only used for purifying water, but also for preventing diseases and promoting fish growth. Another type is the product made of the intestinal anaerobic bacteria (IAB) which helps in reconstruction and regulation of the micro-ecological systems.

5.4 Eradicating the enemies protecting the farmed objects

The enemies of the farmed animals are mainly referring to predating fishes, aquatic insects, amphibians, reptiles and birds. The eradicating of these animals should follow different approaches:

A Control the number of the predating fishes: The normal predating fishes are, snakehead (*Channa argus*), *Siniperca chuatsi*, catfish, *Pseudobagrus fulvidraco*, *Monopterus albus*, *Opsariichthys bidens*. The eradication of the wild predating fishes should be done prior to the stocking of the fingerlings. Necessary measures should be also taken to prevent the inflow of the additional predating fishes when the pond is irrigated. An understanding should be made on the spawning ground and conditions of the predating fishes so that manipulations should be continued to destruct the spawning process. Large size fingerlings stocked are also effective in controlling the predating fishes.

B. Eradicating aquatic insects. The normal insects are *Laccotrephes* sp., *Ranatra chinensis*, *Kirkaladyia deyrouei*, *Hydra vulgaris*, *Cyclestheria*. Some of the insects catch the fish, some eat the fish eggs. Reproduction of these aquatic animals will also consume a great deal of the oxygen and nutrients in the water bodies.

C. Capture of the unwanted animals:

Snakes, water mice, frogs and tadpoles and water birds should be timely removed and driven off. These animals are not only predating but also carriers of the disease pathogens.

5.5 Use of the growth promoters to speed up the growth rate of the fish**A. Application of the growth substances:**

The growth substances are mixed up with the feeds to feed the fish to activate the fish growth, regulate the metabolic rate of the nuclear acid and prevent the expansion and the transmission of the fish diseases. *Undaria pinnatifida* can nicely be used as feed promoter and at the same time it is also used for the treatment of the ulceration of the fish.

B. Rare soil: A certain concentration of the rare soil is applied to inhibit the growth and reproduction of the diseased pathogens. Fish appetite is stimulated, anti-resistance is enhanced. All that is good and effective for the red skin disease, enteritis and gill rot of grass carp.

C. Bio-activated products: The bio-activated products are the substrates of the plants, functioning on the nerve systems and the balance of the internal enzymes for better immunity against the mutation, bacteria and virus.

5.6 An appropriate use of the fish chemicals

A. Right use of the fish chemicals: The fish chemicals should be used not only for the disease prevention and control but also good for the regulation of the metabolisms, improvement of the digestion, promotion of the growth and enhancement of the feed efficiency. The right use of the fish chemicals should be based on the understanding of the needs of the fish. A good understanding

should also be made on the source of the pathogens, obstacles should be created to stop the transmission of any disease. The application of the fish chemicals should be based upon the less pollution, less residues, less toxicants and low cost.

B. An appropriate method in applying the chemicals:

The methods of the chemical application are summarized as the following three types: A. mixture with water. It is diluted in the water for fish to bathe or pond spray. B. Medicated feeds. The medicated feeds should be evenly mixed up with an appropriate concentration. C. Injection.

C. Better quality of the fish chemicals:

The anti-resistance of the fish chemicals has received much attention from the growers. The general recommendation of the fish chemicals should be in an alternative use of different kinds to avoid the resistance of the pathogens. On the other hand, more research needs should be carried out for better quality and more efficiency of the chemicals.

D. Application of the herbs.

The herbs are the traditional methodologies in helping the treatment and prevention of the fish diseases without any residues. It is also served as a health flourisher. In China there have been a good result by using the herbs for the fish treatment.

5.7 Development and application of the fish vaccines

Development of the effective vaccines for the fish is a half way in achieving the success. However, it takes a long time in the research findings. In 1980s, vaccines of SVCV were for marketing in Europe. It is necessary and urgent to develop good quality with a good effect of the vaccines that can be turned for general marketing.

Quarantine, Surveillance and Monitoring of Koi Herpesvirus in Singapore

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Abstract

The Koi industry in Singapore is a sizable one. Thus the potential introduction of any significant disease such as Koi Herpesvirus (KHV) or Spring Viremia of Carp Virus (SVCV), will be of concern to Singapore. Singapore's ornamental fish are imported and exported by traders licensed under the 'Accredited Ornamental Fish Exporters Scheme'. Under this Scheme, the exporters have to get their premises approved according to guidelines set by the Agri-Food and Veterinary Authority of Singapore (AVA), which include the provision of designated quarantine area, fish disease treatment area, and packing area. These approved premises are inspected monthly by AVA inspectors. As part of routine fish disease surveillances conducted by AVA, regular fish samples are taken from each exporter premise once every six months for laboratory examinations, to ensure the absence of any significant diseases. Additional samples are taken for laboratory examinations at greater frequencies should any significant disease outbreaks occur in these premises. Significant results from these surveillances are reported in the Quarterly Aquatic Animal Disease Report (Asia and Pacific Region), which is submitted to the OIE and NACA. Since January 2003, no positive case has been detected to date from AVA's surveillance for KHV in Singapore.

Key words: Koi Herpes Virus, Accredited Ornamental Fish Exporters Scheme, laboratory examinations, surveillances, Aquatic Animal Disease, consignments, compulsory inspection, Quarantine

Introduction

Since reports of Koi Herpesvirus (KHV) as a significant disease of Koi in Israel, Europe and United States in 1998 (Hedrick *et al.*, 2000), and the Koi Mass Mortality Syndrome in Indonesia in June 2002 (Sunarto and Rukyani, 2004), ornamental fish traders dealing with Kois in Singapore have been concerned about this disease. The Agri-Food and Veterinary Authority of Singapore (AVA) has dialogue sessions with Singapore Koi farmers and traders in July 2003, to address their concerns and to promote measures to prevent the import of KHV-infected fish into Singapore. Since reports of KHV outbreak in Japan in October 2003 (Kimiya, 2004), AVA has instituted compulsory inspection, testing and

quarantine of all Koi consignments imported from Japan and Indonesia. Quarantine is for a minimum period of 3 weeks. Koi samples from a particular consignment, or sentinel KHV-free Koi placed together with these imported Koi, are subjected to testing for KHV by tissue culture. Only Koi tested negative for KHV will be released from quarantine. KHV positive Koi consignments will be destroyed, and the premise and equipment disinfected accordingly.

Singapore is known as the ornamental fish capital of the world. Despite the steep competition in recent years, Singapore still remains as a major player in the global business of ornamental fish export. In 2002 Singapore export about 235 million pieces of ornamental fish worth S\$ 74 million (US\$43 million) to more than 70 countries

(Anonymous, 2002). There are 64 ornamental fish farms and 103 ornamental fish exporters in Singapore. The local farms produced about 44% of the total export value of S\$72.8 million (US\$42.3 million) in 2003 (Ling and Lim, 2003). The increase in international trade of ornamental fish over the years poses a great challenge to national authorities in their vigilance against the potential establishment of exotic aquatic diseases in their countries. This has prompted many importing countries to impose more stringent health requirements on the incoming fish with particular emphasis on the specific disease-free status of the originating source. Over the years, AVA has been working closely with the Singapore's exporters in ensuring the Singapore export consignments meeting the increasing demand on fish quality and health status. AVA's quality assurance programmes aim to provide a credible and all-rounded approach towards the promotion of good hygiene and sanitation practices within the premises of the exporters. Emphasis is placed on the exporters' abilities to maintain an established standard of practice and a proper documentation process. AVA monitors the exporters' premises through regular inspection and water and fish samples to ensure compliance and provides technical advice on quality and health management matters. These programmes hence offer a comprehensive assurance of quality and fish health status for the export of Singapore ornamental fish to the global market.

Current Status of Koi Herpes-virus Disease (KHVD) in the Production of Common Carp and Koi Carp

Production of Common Carp

Owing to a very small market demand of common carp, there is no commercial farming of common carp in Singapore. There is limited number of wild common carps thriving in reservoirs and lakes in Singapore. These are for leisure and control of pest, but not for consumption. Hence, there is no harvesting or fishing of common carps activities being carried out in reservoirs and public lakes. Singapore imported less than 50 metric ton of common carps yearly for the last 3 years, mainly from Malaysia for offering in special festivals (Table 1).

Detailed breakdown of import in the past 3 years is summarized as follows: Being low in market demand, there is no export or re-export of common carp's trading activity in Singapore.

Table 1. Import of live common carp in Singapore for 2001- 2003.
Source: Singapore Trade Statistics (2003), Unit: metric tons.

	2001	2002	2003
Malaysia	32.68	43.26	41.03
Hong Kong			0.02
Taiwan	0.50		
Total	33.18	43.26	41.05

Production of Koi Carp

There are 4 fancy Koi farms and about 40 major Koi importers/exporters in Singapore. The activities of Koi farms are mainly involved in import of Koi fingerlings and nursing them into bigger size for local sale or export. Besides growing and nursing of high quality Koi, the farms also provide Koi hotel services for Koi hobbyists. Many Koi hobbyists in Singapore are HDB flat dwellers. They do not have sufficient space for their pet fish, thus they rent pools or ponds from the Koi hotel. Rental ranging from \$100 per month for a pond with a capacity of 10m^3 to \$350 for a pond with 50m^3 . Rental covers daily feeding, maintaining and checking of the Koi during weekdays. Owners are required to provide feeds for daily feedings. They usually spend weekends and public holidays with their pet fish at the Koi hotel. Local production of Koi is negligible. The importers/exporters with holding and quarantine facilities, import Koi from different sources, quarantine them for a period of 3 week or hold for a longer period until the fish grow bigger or are in better shape, then exported to other countries. Singapore imports Koi of different sizes mainly from Malaysia, Japan, Thailand and China. The import figures of Koi for the last 2 years are shown in Figs. 1 and 2. The main supplier of Koi to Singapore is Malaysia which supplied more than 90% of the total quantity imported, followed by Japan (about 2 %) and China (1.5%), (Singapore Trade Statistics, 2003).

Singapore exports about 2.4million pcs of Koi annually to other countries.

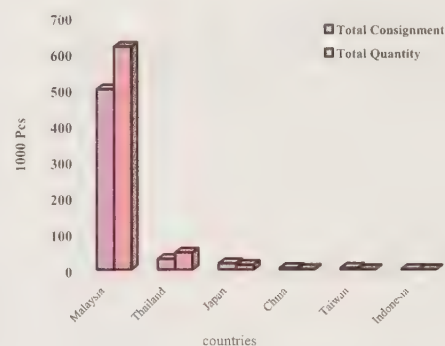
The major importers of Koi from Singapore are United Kingdom, Germany, United States and Malaysia (Figs. 3 and 4).

Export figures for the past 2 years are summarized in Fig. 5.

Fig. 1. Koi imported from various countries in 2002



Fig. 2. Koi imported from various countries in 2003



Koi Herpesvirus Disease (KHVD) of Common Carp and Koi Carp

Singapore has so far effectively controlled KHV from transboundary transmission through quarantine measures. Basically this quarantine process for Koi imported from suspected areas is for a period of 21 days. Therefore, Singapore is free from KHVD.

Fig. 3. Top 10 importing countries of koi from Singapore in 2002

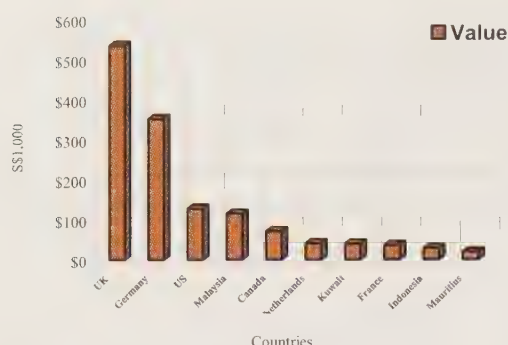


Fig. 4. Top 10 importing countries of koi from Singapore in 2003

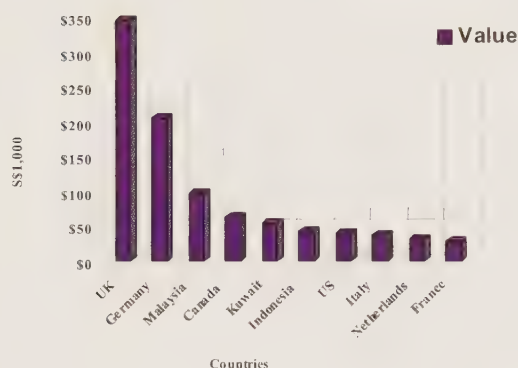
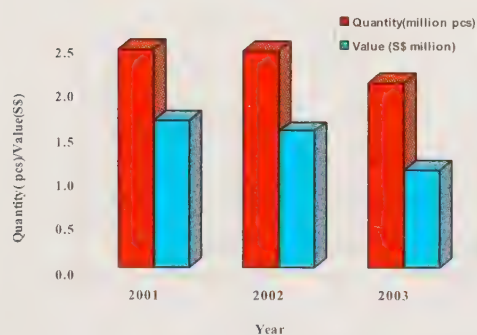


Fig. 5. Koi export statistics for last 3 years (2001 - 2003)



The National Aquatic Animal Health Management Strategy in Singapore

AVA is the only national authority responsible for aquatic animal health management in Singapore. It is empowered by the Fisheries Act (Chapter 111), Animal and Birds Act (Chapter 7) and the

Wholesome Meat and Fish Act (Chapter 349A), which prove the legislative framework for the regulation and monitoring of live aquatic animal trade and health management. Apart from implementation of specific monitoring programmes for the purpose of health certification for live fish trade, AVA also carries out surveillance for significant pathogens and conduct regular surveys and inspections of fish farms and exporters premises.

Fish Health Certification Programme

Certification has become a prerequisite for international movement of aquatic animal and a means of facilitating trade among countries. AVA is the only authority in Singapore to provide fish health certification services to live fish trade and industry. With the ever-increasing live fish consignments being exported from Singapore, certification is primary based on a good reliable system on inspection and monitoring of the fish farms and export premises.

Accredited Ornamental Fish Exporter Scheme

For the purpose of health certification for export or ornamental fish, AVA implements the accredited Ornamental Fish Exporters Scheme to monitor fish health status and sanitation of export premises. The Scheme, initiated in 1983, is to encourage Singapore ornamental fish exporters to export healthy fish that are of high quality through maintenance of high standard of hygiene and sanitation in their export premises. The Scheme enables exporters with good management practices to obtain accreditation for their export premises and obtain health certificates based on their good track record of management practice on hygiene and sanitation, and fish quality rather than through inspection of every consignment. Membership to the Scheme is voluntary and through application by companies that are licensed importers and exporters of ornamental fish. Prior to the approval of

membership, the premises would be subjected to initial screening by AVA, including inspection of the premises and fish stock, water sampling for laboratory examination and checking the records of the fish sales maintained by the company. There must not have any history of fish disease occurrences or any gross mortality in the fish stocks in the export premises during the one month preceding the commencement of membership. A Code of Practice is incorporated into the Scheme to provide guidelines on the management of incoming and outgoing fish, routine care of fish held in the premises and packing of fish for export and maintenance of the packing premises. In addition, members also have their own quality control measures to ensure that only good quality and healthy fish are included in their consignments. More importantly, members must also meet the requirements of the importing countries.

Surveillance and Monitoring Programmes for Aquatic Animal Pathogens

Ornamental fish samples taken from exporters' premises have been screened for Epizootic Haematopoietic Necrosis Virus (EHNV), Infectious Pancreatic Necrotic Virus (IPNV), Infectious Haematopoietic Necrosis Virus (IHNV), Viral Haemorrhagic Septicaemia Virus (VHSV), Spring Viremia of Carp Virus (SVCV), and KHV with negative results to date.

Farm and Export Premises Survey

Surveys of fish farms and exporter premises are carried out on a monthly & quarterly basis by AVA. For these surveys, AVA officers visit the fish farm and audit the farmers/exporters on their farming production methods. In addition, farmers/exporters who encounter disease problems/outbreaks can approach AVA for assistance, either by telephone/fax, email or directly in-person at AVA Services Centres. Disease investigation is carried out by officers from Aquatic Animal Health Branch,

Epidemiology and Surveillance Branch and Aquaculture Branch, who work closely on these reported disease cases. Prompt action is always taken to ensure that farm visits are made and the affected species inspected. Samples are usually taken back to the laboratories for full post-mortem examination and disease diagnosis. Farmers/exporters are then advised on the course of action to take. If treatment is necessary, AVA staff will be on hand to train or guide farmers/exporters on proper treatment procedures. They will also be notified of the results of the investigations and given the necessary advice.

Specific Import and Quarantine Requirements

Besides the compulsory requirement of import licences and permits for all aquatic animals consignments coming into Singapore as described above, AVA imposes specific import and quarantine requirements for certain high-risk consignments coming in from sources where outbreaks of significant diseases are known. In the recent outbreak of KHV around the region, AVA has instituted compulsory inspection, testing and quarantine of all Koi consignment imported from countries with known outbreaks of the infectious disease. Fish are required to be quarantined in an isolated area at the importers' premises for a minimum period of 3 weeks, during which 10 samples from the consignment should be collected and send to the laboratory under Aquatic Animal Health Branch for screening of KHV virus. For the case of high quality and expensive Koi, 10 sentinel Koi cohabiting with these imported Koi for 7 days, are subjected to test for KHV by tissue culture. Only when the test for KHV is negative, will the fish be released from quarantine. KHV positive Koi consignments will be destroyed, and the premises disinfected accordingly. Water from the quarantine area are to be disinfected with chlorine and discharged into the sewage system to prevent KHV from entering into

waterways that lead to reservoirs where there are wild carps.

Development of Good Management Practice for Aquaculture

A Good Management Practice Scheme has been established for ornamental fish farms in Singapore. The Scheme, currently on a trial basis involving participation from some 13 farms, is voluntary and members are required to fulfil certain criteria in good farm management and adhere to a Code of Practice. The Code specifies guidelines on the various aspects of farming such as maintenance of farm premises, water quality management, farm hygiene, husbandry, pond/tank management, feeds and feeding, fish health management, water drainage and treatment, harvesting and post harvest & packing procedures and proper recordings. The farm is subject to regular inspection by AVA to ensure compliance. AVA is also in the process of developing a Good Aquaculture Practice (GAP) Scheme for food-fish farms.

Conclusion

As KHVD is very infectious and causes heavy mortality once succumbed. AVA has imposed all necessary controls to prevent KHV from entering into Singapore. This included a compulsory quarantine of all Koi imported from suspected country for at least 21 days. From the information gained from the International Symposium on Koi Herpesvirus Disease and study trips in Japan, we observed that the KHV problems faced in Japan have been due to the transmission of the virus to carps in lakes and reservoirs whereas KHV in Koi has been effectively contained. Therefore Koi farmers, importers, and exporters are recommended to pay attention the followings:

- (1) Be vigilant to prevent similar disasters that have occurred in other countries.
- (2) Quarantine Koi imported from suspect country in an isolated area for at least 3 weeks prior to obtaining laboratory report.

- (3) Send 10 Koi sample to Aquatic Animal Health Branch, AVA for screening of KHV and generation of the laboratory report.
- (4) Cohabite 10 sentinel Koi with the high quality and expensive Koi for 7 days and send the sentinel Koi to the Aquatic Animal Health Branch for screening of KHV. This has been an innovation from Singapore and was acknowledged by the Symposium in Japan.
- (5) Disinfect water from the quarantine area with chlorine and discharge water into the sewage system to prevent KHV from entering into waterways that lead to reservoirs where there are wild carps.
- (6) Release quarantined Koi for culture or export only upon confirmation of KHV negative results from the Aquatic Animal Health Branch.

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Thailand's Current Quarantine Status on Aquatic Animal Diseases

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Abstract

An awareness of aquatic animal diseases spread through an international trade has been increasing since the first edition of OIE on Aquatic Animal Health Code in 1995. How to control the diseases through an international trade and a development of national strategy for controlling the diseases had been discussed in great details among representative from 21 Asian governments during a three years (1998-2000) seminar and workshop program organised by NACA. As part of the region, the strategic plans for aquatic animal health management in Thailand have been developed. Presently, Thailand has over 21 import/export regulations announced through Emergency Decree, Royal Decree, Ministerial Regulation, Notification or Rule to control the movement of certain aquatic animals both from importation and exportation. The aquatic animal disease quarantine enforcement is based on the Animal Epidemic Act B.E. 2499 (1956) and Fishery Act B.E. 2490 (1947). The Department of Fisheries has developed an importation of live aquatic animals under quarantine enforcement framework, which is based on disease control program and disease surveillance. The quarantine measures include pre-arrival, arrival at the entry port and post-arrival of aquatic animals that will be discussed. Koi herpesvirus disease surveillance system has been in place since August 2002 and Thailand is still free.

Key words: aquatic animal quarantine, koi herpesvirus, KHVD, trans-boundary disease, import requirement

Introduction

Movement of aquatic animals has been generally recognized as a high risk of transferring the diseases and pathogens from one area to another. A review from scientist indicated that an international fish trade has spread diseases to many countries for years (Håstein, 2000). In case of Thailand, an introduction of Chinese carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idellus*, *Aristichthys nobilis*) for food fish culture in the past also introduced *Lernae* parasites into the aquatic ecosystem.

Importation of ornamental fishes also introduced many new pathogens such as *Hexamita* and *Tetrahymena*. The occurrences of ranavirus infection in tadpole, frog and fish cultured in Thailand might come from the healthy aquatic animal carriers that imported into the country. Some pathogens have a wide host ranges including food fish and ornamental fish. The susceptible hosts exhibit disease clinical signs and death. However, the resistance host or the disease-recovered fish will possible serve as reservoir or carriers of the parasites. An introduction of shrimp from China caused WSSV

epidemic. An illegal importation of Pacific white shrimp caused TSV outbreaks in Thailand. Solving problems of trans-boundary pathogens or diseases in a systematic way, the *“Thailand National Strategy for Control of Aquatic Animal Diseases”* has been developed after the seminar among staffs from Department of Fisheries (DOF), Department of Livestock Development (DLD), University, Private Sectors and Farmers in Bangkok in May 2001. The strategic plans are listed as follows; (1) law and legislation, (2) import/export regulation, (3) disease surveillance, monitoring and control systems, (4) aquatic animal diseases; research & development, (5) diagnosis units; capability building, (6) technology/knowledge transfer, (7) public awareness, (8) contingency plan to control disease outbreak and (9) funding support. The strategic plans have been implementing with a good progress.

Ways forward to control aquatic animal diseases

Law and legislation for controlling aquatic animal diseases have been developed under the existing the Animal Epidemic Act in June 2003. Twenty seven aquatic animal diseases are listed under this Act to be controlled in the country. A joint working group between DOF and DLD has been appointed and this group has been working in details on how to apply the law to control the aquatic animals and their diseases. Beginning on July 1, 2004, this Animal Epidemic Act has been implementing to control aquatic animal diseases.

Current Quarantine Measures

The Department of Fisheries (DOF) has established a strategy plan to control trans-boundary movement of aquatic animal diseases using quarantine measures. Fish Quarantine Inspector conducts inspection services at the port of entry. All live aquatic animal shipments are inspected at the port,

then they will be sent to quarantine for at least 15 days at the quarantine facility that passed the standard biosecure. During the quarantine period, Fish Health Inspector will visit and conduct health inspection of the imported animals at the quarantine facility. The Fish Health Inspectors are Fishery Biologist or Fish Pathologist assigned by DOF. We use diagnosis Level III for disease diagnosis of the imported aquatic animals. Steps for live aquatic animal importation into Thailand summarized as follows;

- Pre-importation: facilities of Thai aquatic animal farms or companies have to achieve the quarantine standard of the DOF before receiving an import permit.

- Arrival of aquatic animals and their gametes at the port: the imported animals and their gametes must be accompanied with Certificate of Origin and Health. The fish will be subjected to quarantine at the certified quarantine areas of the importing farms or companies.

- Post-importation: aquatic animals will be quarantined for at least 15 days. Fish health inspectors will examine the animals for diseases listed in the OIE, koi herpesvirus and other contagious pathogens. If serious pathogens are found, the animals and their gametes will be destroyed without compensation. If the fish are free from listed diseases, the importation procedures are completed. However the fish still need to be kept in the quarantine until 15 days quarantine period.

Requirements for Importation

The Department of Fisheries (DOF) has set up a new regulation to prevent and control aquatic animal diseases through importation. The imported live aquatic animals subject to be quarantined at the approved quarantine zone of the importing companies for at least of 15 days. Health inspectors will inspect the animals in the quarantine zone and will take samples for laboratory tests. A health certificate must be presented at the port of entry together with the aquatic animal

shipment. The health certificate must be issued by competent authority, signed by veterinarian or authorized officer and contained information as follows;

- 1. Name and address of consignee.
- 2. Name and number (scientific and common name) of aquatic animals.
- 3. Origin of the aquatic animals exported.
- 4. The aquatic animals must come from a country, a zone or a farm establishment where they are submitted to a health supervision set up to operate according to the procedures described in the “*Diagnostic Manual for Aquatic Animal Diseases*” from *Office International Des Epizooties* (OIE) and that this country, zone, or farm establishment is recognized officially

- unaffected by the OIE listed diseases. If the test methods of any diseases are not designated in most recent edition of the OIE Diagnostic Manual, test methods of the disease which having been published in international science journals shall be used and must be states in the certificate.
- 5. The exported animals must not come from the sources that had an un-usual mortality during the previous three months, which the causation could not be explained.
 - 6. Before exportation, the animals must be quarantined for 7-10 days and treated with chemicals to remove all external parasites.
 - 7. The exported animals must certify as indicated in the following table (Table 1);

Table 1. Types of l diseases or pathogens needed to be certified and stated in the health certificate.

Type of aquatic animals and their gametes to be exported	Type of diseases or pathogens
Freshwater fish	-Epizootic haematopoietic necrosis virus -Iridovirus disease or Ranavirus disease -Viral haemorrhagic septicaemia -Spring viremia of carp virus -Koi herpesvirus disease
Marine & estuarine fish	-Red sea bream iridovirus disease -Viral encephalopathy and retinopathy
Mollusks	-Bonamiosis -MSX disease -Marteiliosis -Mikrocytosis -Perkinsosis
Crustaceans	-Taura Syndrome virus -White spot syndrome virus -Yellowhead virus -Infectious hypodermal and haematopoietic necrosis virus
Amphibians	-Iridovirus disease or Ranavirus disease -Epizootic haematopoietic necrosis virus
Reptiles	-Iridovirus disease or Ranavirus disease -Poxvirus

Background of common carp and koi carp cultured in Thailand

There were 3 culture systems for common carp, pond, ditch and cage systems. For pond culture system, farmers normally raised the carp with other fish species (poly-culture system) or with other animals (integrated culture system). For paddy-field culture system, the farmers cultured the carp in the rice paddy field during the rice crop. For ditch culture system, carps were cultured in the ditch in the fruit or vegetable plantation. According to Fishery Statistics Analysis and Research Group (2001), Freshwater aquaculture farms recorded were 389,374 farms (pond culture 355,624 farms, paddy field culture 14,829 farms, ditch 7,165 farms, cage culture 1,207 farms) in Thailand. The total freshwater aquaculture production and value were 279,696 ton and 9,279.8 million Baht. There were ~17,465 common carp culture farms (pond culture 15,693 farms, paddy-field culture 1,723 farms, ditch culture 49 farms) recorded in year 2001. 90% of common carp farms were pond culture system and there was no record of common carp cage culture in Thailand in 2001. The common carp production and value during year 2001 were 4,773 ton (pond culture 4,026 ton, paddy-field culture 736 ton, ditch culture 10 ton, cage culture 0.5 ton) or 146,658 Baht in value.

The carp can be found in the wild, canals and rivers. There was no statistic value for the wild caught carp as the quantity and value were very low.

Generally, fish farmers obtained the carp seeds from government hatcheries or private hatcheries. Thailand introduced common carp from China about 100 years ago. There was no record of common carp exportation out of Thailand. Common carps were mainly used for local Chinese people consumption and their pituitary glands were used for artificial fertilization in fish hatcheries. In the past or ~20 years ago, the pituitary glands of the common carp were highly demanded

from fish hatcheries. Since the synthetic hormones gave similar stimulation on gonad maturation, the demands for pituitary gland of the carp reduced.

Koi carp production in Thailand has been increasing during the past 3-5 years. Thailand is located in tropical zone and the average water temperatures are warm (28-32°C) through out the year. Kois rapidly grow and have a low risk against cold-water diseases such as Spring viremia of carp virus (SVCV) and koi herpesvirus (KHV). There is no record for koi production in the country. Koi carps are cultured in earthen pond, concrete pond and cage. The koi brooders are from local source as well as imported source from Japan. Thailand exports koi to many countries. Since the outbreak of SVCV and KHV in some countries in the region, koi exportation of Thailand is getting high.

Koi Herpesvirus Disease (KHVD) Status

Thailand started the KHVD surveillance program since August 2002 or just a few months after the first outbreak of koi mass mortality in Indonesia. Since then Thailand is still free from KHVD. The Department of Fisheries (DOF) also developed a rapid response or gave a high priority to investigate any disease cases reported by the fish farmers or by the provincial fishery officers regarding to the mass mortality or unusual death of kois or common carps. At the moment, we do KHVD survey using virus isolation in KF-1 and BF-2 cells and PCR detection.

Summaries

The Department of Fisheries has been intensively working on *Thailand National Strategy for Control of Aquatic Animal Diseases*. Several working groups have been assigned to develop rules and implementation systems. Training workshops have been given to different groups of Fishery Officers.

The DOF called a few meetings with importers and exporters for understanding the necessary of the new regulations. Starting on July 1, 2004, all importations and exportations of aquatic animals and fishery products are regulated under the new rules of the Animal Epidemic Act for strengthening disease control. Starting on November 1, 2004, all importations of aquatic animals must follow the above three steps of importation that has been already notified to the WTO (notification # G/SPS/N/THA/114 dated September 17, 2004).

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Survivability of Fish Pathogenic Viruses in Environmental Water, and Inactivation of Fish Viruses

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Abstract

Survival of three salmonid viruses and two marine fish viruses in fish rearing water or coastal sea water were observed at 0, 5, 10 and 15 °C for 7 or 14 days. Interaction between viruses and microorganisms present in the rearing water was observed. Infectious pancreatic necrosis virus (IPNV) and fish nodavirus (BF-NNV) were stable in waters used at every temperature tested for 14 days, but it was observed that, for infectious hematopoietic necrosis virus (IHNV), Oncorhynchus masou virus (OMV), and hirame rhabdovirus (HIRRV), as the temperature increased, the loss of infectivity also increased. When IHNV and OMV were suspended in filtrated and autoclaved rearing water, infectivity was reduced in comparison with the untreated water. Subsequently, adsorption of IHNV to mud or small particles was studied. IHNV adsorbed to several clays (kaolin, bentonite, Japanese acid clay) and diatomaceous earth in sterilized water with a wide range of pH (5-11) at concentrations of 1, 10, and 100 mg/mL. Except for bentonite, infectivity of clay-adsorbed IHNV persisted for at least 9 weeks. The clay-adsorbed IHNV also persisted in infectivity to rainbow trout *Oncorhynchus mykiss*, causing cumulative mortality rates of more than 73 %. Then, inactivation effects of UV irradiation, ozonization, and electrolyzation of water were studied against six fish rhabdoviruses, three fish herpesviruses, one fish birnaviruses, one fish iridovirus, and one fish nodavirus. Six rhabdoviruses, three herpesviruses, and lymphocystis disease virus were found to be sensitive to UV irradiation, ozonization, and electrolyzation. Susceptibility of IPNV, chum salmon virus (CSV), and BFNNV to UV was found to be low. IPNV and CSV were low sensitive to ozonization and electrolyzation. Virucidal effects of six kinds of disinfectants were examined against OMV, IPNV, IHNV, and HIRRV at 15 and 20°C for 30 sec and 20 min. At 15°C for 20 min, minimum concentrations showing 100 % plaque reduction of viruses tested by iodophore, sodium hypochlorite solution, benzalconium chloride solution, saponated cresole solution, formaldehyde solution, and potassium permanganate solution were 40, 50, 100, 100, 3500, and 16 ppm, respectively.

Key words: fish virus, survivability, environmental water, inactivation, disinfectant, UV, ozonization, electrolyzation

Introduction

The knowledge of survival of fish viruses in the fish rearing water and interaction of viruses and other microorganisms in aquatic environments are critical for establishing the control methods of fish viral diseases. Several previous reports have described the inactivation of viruses in natural water and sludge which at certain times seem to be related to microorganisms inherent in these environments (Kamei *et al.*, 1987 a,b, Kamei

et al., 1988 a,b, Kimura *et al.*, 1988). Disinfection of the water for aquaculture is also critical for preventing the introduction and spread of infectious disease. A pathogen free water source is essential for success in aquaculture. Surface waters commonly used in aquaculture come from coastal waters or rivers and may contain some fish pathogens and such open water supplies should not be used without treatment. Disinfection of wastewater before discharging is necessary to avoid the pathogen contamination in the

environment (Kasai *et al.*, 2002, Yoshimizu and Kasai 2002). In this paper, we introduce the survival of fish viruses in the fish rearing water and virucidal effect of ultraviolet (UV) irradiation, ozonization, and electrolyzation of water. Additionally, virucidal effects of disinfectant against fish viruses are introduced.

Materials and Methods

Survivability of fish viruses in environmental water

Survival of three salmonids viruses; infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and *Oncorhynchus masou* virus (OMV) in fish rearing water, de-chlorinated city water, double-distilled water, and Hanks' balanced water (HBSS) (Yoshimizu *et al.*, 1986), two marine fish viruses; hirame rhabdovirus (HIRRV) and fish nodavirus (barfin flounder nervous necrosis virus; BF-NNV) in coastal sea water, filtrate sea water, and autoclaved sea water, were observed at 0, 5, 10 and 15°C for 7 or 14 days. Interaction between viruses and microorganisms present in the rearing water was observed.

Adsorption of IHNV to mud

Adsorption of IHNV to sea sand, Japanese acid clay, diatomaceous earth, kaolin, bentonite, quartz sand, chitin, cellulose powder, ion exchange hydrophobic Toyopearl and Cellulofine, alundum, active carbon, silica gel glass, plastic, and bacterial cells was studied (Yoshinaka *et al.*, 2000). Infectivity of clay-adsorbed IHNV was observed using a cell line and rainbow trout *Oncorhynchus mykiss*.

Inactivate effects of UV irradiation, ozonization, and electrolyzation

Inactivation effects of UV irradiation, ozonization, and electrolyzation of water were studied against six fish rhabdoviruses; IHNV, HIRRV, pike fry rhabdovirus (PFRV), spring

viremia of carp virus (SVCV), eel virus from America (EVA), and eel virus from Europe X (EVEX), three fish herpesviruses; OMV, *Herpesvirus salmonis* (*H. salmonis*), and channel cat fish herpesvirus (CCHV), fish birnaviruses; IPNV, fish reovirus; chum salmon virus (CSV), fish iridovirus; Japanese flounder lymphocystis disease virus (JF-LCDV), and fish nodavirus (BF-NNV) (Kasai *et al.*, 2002).

Virucidal effects of disinfectants

Virucidal effects of six kinds of disinfectants; iodophore, sodium hypochlorite solution, benzalconium chloride solution, saponated cresole solution, formaldehyde solution, and potassium permanganate solution were examined against OMV, IPNV, IHNV, and HIRRV at 15 and 20°C for 30 sec and 20 min (Hatori *et al.*, 2003). Virucidal effects were measured by plaque reduction methods (Kamei *et al.*, 1987c).

Result and Discussion

Survivability of fish viruses in environmental water

IPNV and BFNV were stable in all kinds of waters used at every temperature tested for 14 days but it was observed that for IHNV and HIRRV, as the temperature increased, (Figs. 1 and 2).

OMV was labile even in HBSS and inactivated rapidly as the temperature was raised. It was not survive at 5 to 7 days later in the fish rearing water. When IHNV and OMV were suspended in filtrated and autoclaved rearing water, infectivity was reduced in comparison with the untreated water (Fig. 3) (Yoshimizu *et al.*, 1986).

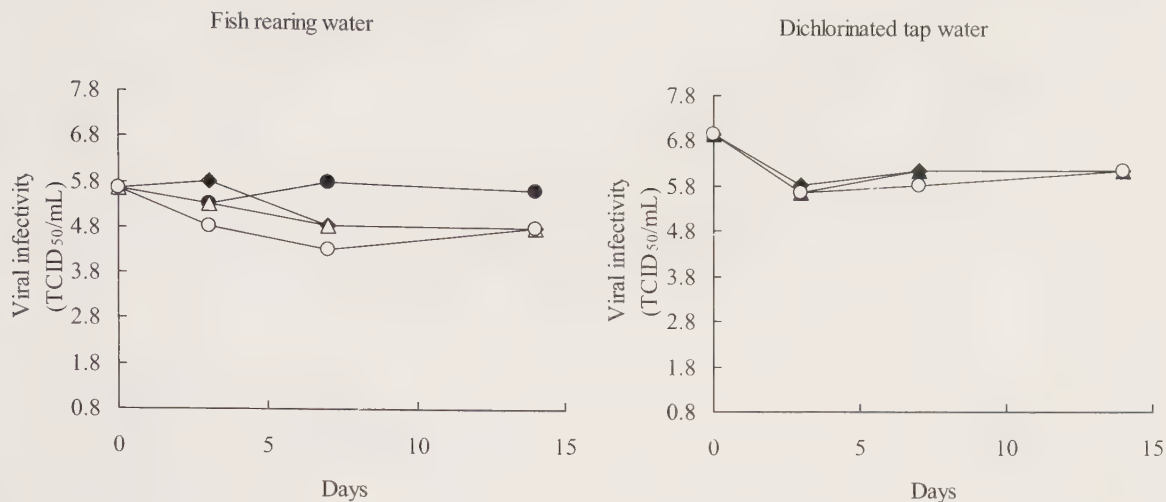


Fig. 1. Survival of IPNV in fish rearing water at 0, 5, 10 and 15 °C.
● ; 0°C, ◆ ; 5°C, ▲ ; 10°C, ○ ; 15°C.

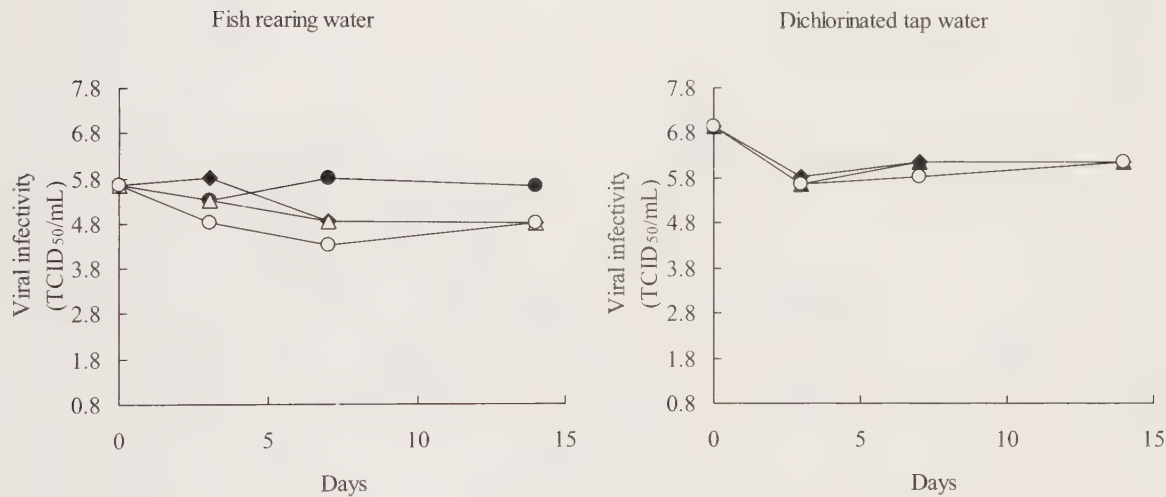


Fig. 2. Survival of IHNV in fish rearing water at 0, 5, 10 and 15 °C.
● ; 0°C, ◆ ; 5°C, ▲ ; 10°C, ○ ; 15°C.

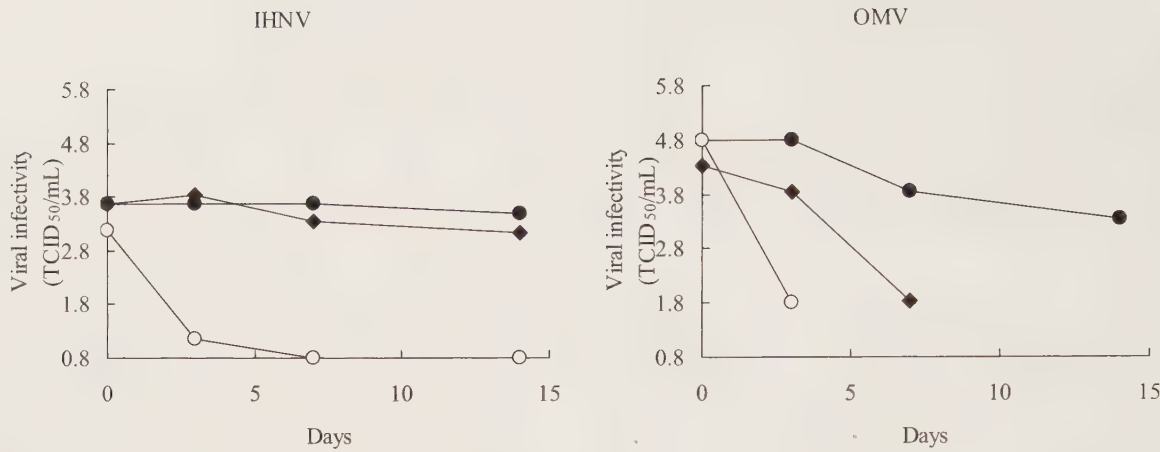


Fig. 3. Survival of IHNV in fish rearing water at 0, 5, 10 and 15 °C.

● ; Autoclaved, ◆ ; Filtered (0.22 μm), ○ ; Non-treated,
▼ ; Under the detection limit.

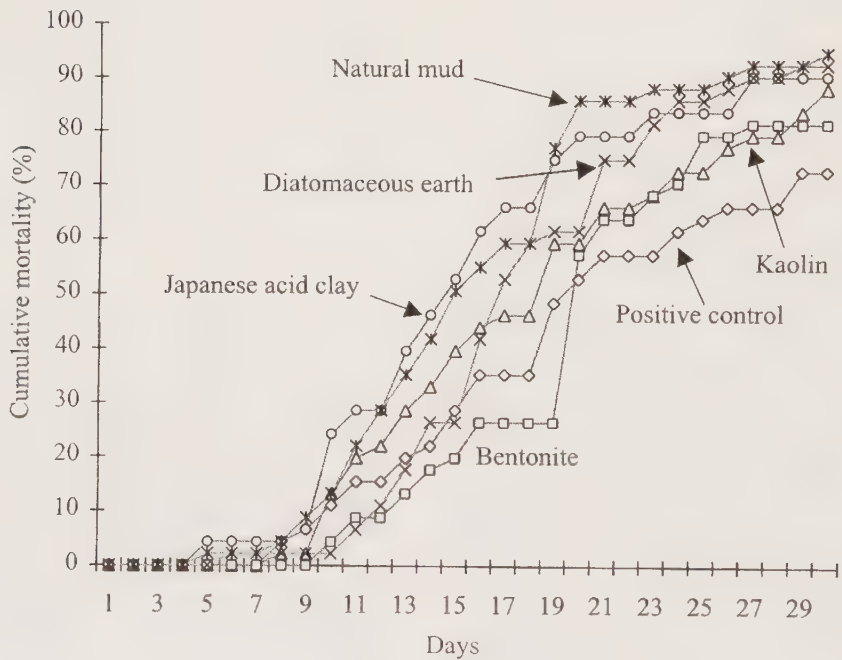


Fig. 4. Cumulative mortality of rainbow trout with bath challenge of IHNV absorbed to solid.

Representative isolates *Pseudomonas fluorescence* strain 46NW-04 has been proven to have the ability to produce anti IHN virus substance.

bentonite, infectivity of clay-adsorbed IHN virus persisted for at least 9 weeks. The clay-adsorbed IHN virus also persisted in infectivity to rainbow trout *Oncorhynchus mykiss*, causing cumulative mortality rates of more than 73 % (Fig. 4). The results suggest that IHN virus adsorbed to naturally occurring substances in various aquatic environments may provide a source of infection for susceptible fish inhabiting these environments (Yoshinaka *et al.*, 2000).

Adsorption of IHN virus to mud

IHN virus adsorbed to several clays (kaolin, bentonite, Japanese acid clay) and diatomaceous earth in sterilized water with a wide range of pH (5-11) at concentrations of 1, 10, and 100 mg/mL (Table 1). Except for

Table 1. IHN virus adsorption onto suspended solids.

Samples	Virus adsorbed*		Virus adsorbed
bentonite	>99.9	polystyrene	44.8
diatomaceous earth	>99.9	polyethylene	83.2
kaolin	>99.9	polyvinyl chloride	83.2
Japanese acid clay	>99.9	ion exchange hydrobic (Cellfine)	44.8
active carbon	99.8	ion exchange hydrobic (Toyoparl)	44.8
quartz sand	0	chitin	83.2
seasand	0	cellulose powder	44.8
glass beads	0	bacteria IC-4	90.0
craft glass pink	69.4	bacteria IE-1	90.0
craft glass yellow	69.4	bacteria IE-2	99.0
craft glass black	90.0	bacteria IG-1	98.2
craft glass orange	83.2	bacteria 2FI6	99.4
craft glass green	69.4	bacteria 46NW04	98.2
alundum	44.8		
polypropylene	44.8		

*: (1-supernatant titer / original IHN virus titer) x 100.

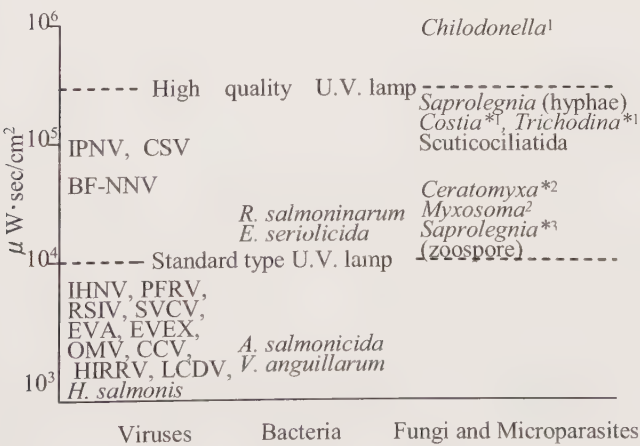


Fig. 5. UV susceptibility of fish pathogens.

*1: Vlasenko, M.I. (1969), *2: Hoffman, G.L. (1974), *3: Normandeau, D.A. (1968).

Inactivate effects of UV irradiation, ozonization, and electrolyzation

Six rhabdoviruses, three herpesviruses, and JF-LCDV were found to be sensitive to UV irradiation (99 % infectivity reduction dose $ID_{99} = 10^3 \mu W \cdot sec/cm^2$), ozonization ($ID_{99.9}$ = total residual oxidants; TROs, 0.5 mg/L for 15 sec), and electrolyzation ($ID_{99.9}$ = Hypochlorite, 0.3 mg/L for 1 min).

Susceptibility of IPNV, CSV, and BFNNV to UV were found to be low, and ID_{99} was measured $10^5 \mu W \cdot sec/cm^2$ (Fig. 5). IPNV and CSV have low sensitivity to ozonization and electrolyzation, and $ID_{99.9}$ was measured 0.5 mg/L TROs for 30 sec and 0.5 mg/L (Tables 2 and 3), hypochlorite for 1 min, respectively (Kasai *et al.*, 2002).

Table 2. Effects of total residual oxidants (TROs) concentrations produced by ozonization of seawater on fish pathogens.

Pathogens	TRO concentration (mg/L)	Treatment time (sec)	Reduction (%)	Initial number (Log. TCID50/mL)
IPNV	0.5	60	>99	4
CSV	0.5	60	>99	4
YTAV	0.5	60	>99	4.3
IHNV	0.5	15	>99	4
HIRRV	0.5	15	>99	5.5
OMV	0.5	15	>99	3
<i>V. anguillarum</i>	0.5	15	>99.9	5.6
<i>E. serioricida</i>	0.5	15	>99.9	5.8
<i>A. salmonicida</i>	0.5	15	>99.9	5.1
<i>A. hydrophila</i>	0.5	15	>99.9	4.6
<i>E. coli</i>	0.5	15	>99.9	6.5
Scuticociliatida	0.8	30	>99.9	5.3

Table 3. Chlorine concentration required to reduce 99.9% of viable bacteria or virus infectivity in 1 minute treatment.

Pathogens	Chlorine concentration (mg/L)
<i>V. anguillarum</i> NCMB 6	0.07
<i>A. salmonicida</i> ATCC 14174	0.06
<i>E. coli</i> O-26	0.14
YTAV Y-1	0.45
HIRRV 8401H	0.34

Table 4. Minimum concentration of disinfectants to show the 100% plaque reduction of OMV, IHNV, HIRRV and IPNV.

Disinfectant	Time	Concentration (ppm)		Concentration (ppm)		Concentration (ppm)		Concentration (ppm)	
		OMV		IHNV		HIRRV		IPNV	
		0°C	15°C	0°C	15°C	0°C	15°C	0°C	15°C
Iodophor	30 S	40	40	100	100	100	100	200	100
	20 min	40	40	100	100	100	100	100	100
Sodium hypochloride	30 S	50	50	50	50	50	50	100	100
	20 min	1	1	50	10	50	10	100	100
Benzalkonium chloride	30 S	100	100	200	200	200	1000	ND*	ND
	20 min	100	100	200	100	100	100	ND	ND
Saponated cresol	30 S	100	100	500	100	1000	500	5000	5000
	20 min	100	100	100	50	500	500	ND	ND
Formaldehyde	30 S	700	700	3500	3500	3500	3500	3500	3500
	20 min	700	350	3500	3500	3500	700	3500	700
Potassium permanganate solution	30 S	16	16	158	158	158	158	32	32
	20 min	16	16	32	16	16	16	16	16

Virucidal effects of disinfectants

At 15°C for 20 min, minimum concentrations showing 100 % plaque reduction of viruses tested by iodophore, sodium hypochlorite solution, benzalkonium chloride solution, saponated cresole solution, formaldehyde solution, and potassium permanganate solution were 40, 50, 100, 100, 3500, and 16 ppm, respectively (Table 4) (Hatori *et al.*, 2003). Koi herpesvirus, the causative agent of mass mortalities of koi and common carp, is a newly isolated virus in Israel, United States, Europe and Asian countries including Japan. Wild resource of carp is also damaged by KHV, thus it's important to monitor the survivability of KHV in the lake and river to control the KHV disease. Although survivability of KHV in the ambient water has not been studied, KHV is considered to be difficult to keep its infectivity in natural environment because this virus belongs to the Herpesviridae same as OMV. With the results of our recent study, KHV was inactivated in the waters and/or mud collected from Lake Kasumigaura during 3 days. While KHV is unstable in the environmental conditions, it shows strong pathogenicity to koi and common carp. Therefore it's necessary to establish an effective control method.

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The Present State of Carp Fisheries and Aquaculture in Japan

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Abstract

Carp (*Cyprinus carpio*), which is thought to have originated from central Asia, is the world's oldest aquacultured fish. In Japan, farmers have cultured carp in paddy fields for nearly two thousand years, particularly in inland areas, such as in Nagano, Gunma and Akita Prefectures, which have been traditionally characterized by a lack of animal protein resources. Since the 1960s, carp production, particularly in Lake Kasumigaura in Ibaraki Prefecture, has been accelerated by new developments in artificial feed and net-pen aquaculture technology. Culture levels peaked in 1977, when total annual production was about 30,000 tons. Subsequently, with the diversification of people's food preferences and product availability, total production decreased gradually in accordance with lower demand and depressed prices. As a consequence, total annual production of cultured carp reached a low of 9,949 tons in 2001.

While, nishikigoi (koi) have been bred as ornamental fish since the Edo Era in the 18th century. Nowadays, koi shows and contests are important activities inside of Japan, and koi traded is conducted throughout the country. International trading is also being actively promoted, and these fish are well-known and admired in many foreign countries. Nishikigoi farms are located in many areas of Japan; 48% of the farms are found in Niigata Prefecture, followed by Gifu (8%) and Hiroshima (6.5%) Prefectures.

Key words: carp, nishikigoi (koi), Lake Kasumigaura, KHV infection

Introduction

The Koi Herpes Virus (KHV) disease that occurred in the Lake Kasumigaura area of Ibaraki Prefecture in October 2003 destroyed most of the cultured common carp present in the lake, and KHV infection was subsequently induced into many new locations such as carp culture ponds and natural rivers in association with transportation of carp from Lake Kasumigaura. One of the reasons for which this large-scale infection of KHV occurred so rapidly in Japan is that the supply of carp seeds depended mostly upon the Lake Kasumigaura region, and KHV disease first arose there. In order to further our

understanding of the current status and problems relating to KHV in Japan, in this manuscript, the history and present state of carp fisheries and aquaculture are reviewed.

History of common carp production in Japan

Carp (*Cyprinus carpio*), which is thought to have originated from central Asia, is the world's oldest aquacultured fish. In Japan, farmers have cultured carp in paddy fields for nearly two thousand years, particularly in inland areas, such as in Nagano, Gunma and Akita Prefectures, which have been traditionally characterized by a lack of animal protein resources.

After World War II, paddy field aquaculture declined with the beginning of wide-spread usage of pesticides, while at the same time, irrigation reservoir aquaculture and high density flow-through pond aquaculture became predominant. As a result, carp production began to increase in response to increasing demands. Since the 1960s, carp production, particularly in Lake Kasumigaura in Ibaraki Prefecture, has been accelerated by new developments in artificial feed and net-pen aquaculture technology. Culture levels peaked in 1977, when total annual production was about 30,000 tons (Fig. 1).

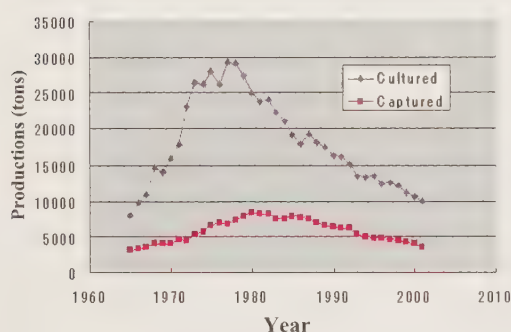


Fig. 1. Changes in the productions of carp in Japan. Data are from “Fisheries Agency, Fisheries Statistical Indices, 2001”.

Subsequently, with the diversification of people’s food preferences and product availability, total production decreased gradually in accordance with lower demand and depressed prices. Total annual production of cultured carp reached a low of 9,949 tons in 2001 (Figs. 1 and 2). However, because of deeply-rooted local needs and cultural preferences, carp remains the third largest target of freshwater aquaculture in Japan following eel and trout. Production based on capture fisheries has fluctuated similarly, reflecting the trend observed with aquaculture production, and was 3,558 tons in 2001. At present, 52% of total carp aquaculture production and 17% of the related seed production is dependent on the Lake Kasumigaura area, and these products are

supplied throughout Japan (Fig. 2).

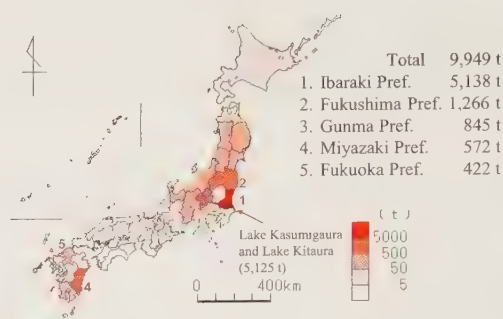


Fig. 2. Carp aquaculture production in 2001. Data are from “Ministry of Agriculture, Forestry and Fisheries, Annual Report of Statistics of Fisheries and Aquaculture Productions in 2001”, 2003 (issued).

Forms of carp aquaculture

There are five major types of carp aquaculture system as follows.

(1) Paddy field (rice field) aquaculture

This is an extensive aquaculture system in which carp are cultured with rice in paddy fields. Recently, this form of aquaculture has declined, except in some cases or in restricted areas, as mentioned above.

(2) Irrigation reservoir aquaculture

In this system, carp are fed and cultured in irrigation reservoir ponds used for agriculture. One-year-old carp fry approximately 100g in body weight are transferred into the reservoir from fish seed culture ponds from April through May, and the fish are reared to market size of approximately 800g by harvest time in autumn. This aquaculture system is usually implemented by private companies or local aquaculture cooperatives mainly in Fukushima and Gunma Prefectures, which have produced as much as 1,266 and 576 tons in 2001, respectively. Feed conversion efficiency and survival rates are approximately 60% and 70%, respectively.

The total annual production from this

system exceeded 10,000 tons in the 1970's, but has lately declined to 2,221 tons in 2001.

(3) Still water pond aquaculture

This is an aquaculture system in which carp are fed in artificial culture still water ponds; 576 tons were produced mainly in Toyama Prefecture in 2001.

(4) High-density flow-through pond aquaculture

This is an intensive and high productive aquaculture system in which carp are fed and cultured in flow-through ponds under high density conditions using clean running river water. This form of aquaculture has been developed in areas where large quantities of clean river water can be easily utilized, such as Fukuoka, Gunma and Nagano Prefectures, and production was 372, 269 and 180 tons, respectively, in 2001. In the flow-through pond, 300 – 500 l / sec of running water is provided, maximum productivity is approximately 300 kg / m², and the quality of the carp meat is high. However, as the oxygen consumption of fish is also enhanced, the feed conversion rate is low as 40%, such that production costs are relatively high.

This aquaculture system has been deeply linked with the silk industry; this is because silk worm pupa can serve as an important component of low cost feeds. In Gunma and Nagano Prefectures, flow-through pond aquaculture has developed together with the silk industry, but more recently, production has decreased in association with the decline of silk production. As a result, the center of carp production has shifted to net-pen aquaculture in the Lake Kasumigaura area.

The total annual production of this aquaculture system was 1,188 tons in 2001.

(5) Intensive net-pen aquaculture

This aquaculture technology was developed by the Miyazaki Prefectural Fisheries Station in 1951 as an aquaculture system which can handle large-scale, intensive carp production at low costs. In the

system, the feeding conversion efficiency and survival rates are approximately 70% and 90%, respectively. This form of aquaculture has been developed in large lakes such as Lake Suwa in Nagano Prefecture and Lake Kasumigaura in Ibaraki Prefecture since the 1960's, but the major production is concentrated in the Lake Kasumigaura area at present. Annual production in 2001 was 5,125 and 500 tons in Ibaraki and Miyazaki Prefectures, respectively.

Utilization of common carp

Carp is majorly utilized as a food fish, and is consumed in fresh, chopped, and processed forms. Carp is particularly popular as an ingredient in local special dishes or seasonal celebration dishes. Since efforts for stock enhancement must be conducted in inland fisheries as dictated by law, the demand for fish seeds is also quite high; local fisheries cooperatives have a responsibility to release seeds into lakes and rivers as well as to conduct aquaculture. Carp originating from seed release are targets of recreational fishing as well, and local fisheries cooperatives managing fishing sites can collect fishing fees from anglers. Additionally, there are private fishing ponds where recreational fishermen pay a fee to fish for carp. In addition, carp and koi are often released into park ponds, urban river areas, or sightseeing locations for purposes of landscape conservation and beautification. Carp can also be found in many private ponds.

Ornamental carp (koi) production

Nishikigoi (koi) have been bred as ornamental fish since the Edo Era in 18th century Japan. The difference between nishikigoi aquaculture and that of common carp is that the breeding strain is very important and the highest quality individuals are extremely expensive. Fish seeds produced in breeding and nursery ponds are reared in culture ponds, and strain selection is

conducted repeatedly in order to discriminate choice individuals. The selected fish are thereafter traded at local auction markets.

In this way, koi shows and contests are important activities in Japan, and the koi trade is conducted throughout the country. International trading is also being actively promoted, and these fish are well-known and admired in many foreign countries.

Nishikigoi farms are located in many areas of Japan; 48% of the farms are found in Niigata Prefecture, followed by Gifu (8%) and Hiroshima (6.5%) Prefectures (Figure 3). Since Niigata has been a rice agricultural area, paddy field aquaculture of nishikigoi has developed historically.

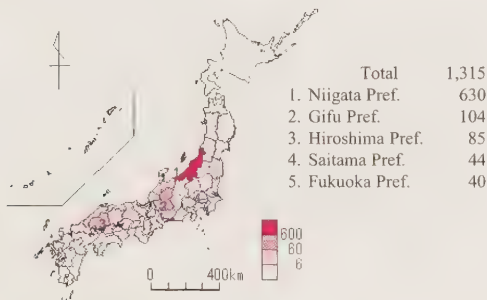


Fig. 3. Number of nishikigoi (koi) farms in Japan. Data are from “Ministry of Agriculture, Forestry and Fisheries, Statistics on Inland Water Fisheries. The 10th Fisheries Census in 1998, Vol. 7, 2000.”

Conclusion

At present, a large portion of carp production, e.g., 52% of total aquaculture production and 17% of total seed production depends upon two particular areas, being Lake Kasumigaura and Lake Kitaura, and this is based on economic and social trends. Therefore, the transportation of carp is often conducted without being in the confines of a single water system. The production and circulation of nishikigoi is smaller in scope than that of common carp, but intra- and international trade and transportation of nishikigoi is being actively promoted. These trends are significantly correlated with the outbreak of KHV infection that is now occurring in Japan. The transportation and handling of carp should be conducted more prudently, observing the regulations of the World Organisation for Animal Health (OIE).

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Diagnosis of Koi Herpesvirus (KHV) Disease in Japan

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Abstract

The koi herpesvirus (KHV) disease was designated as a “Specific Disease” in a Japanese law amended on June 30, 2003. The Ministry of Agriculture, Forestry and Fisheries officially announced the first case of KHV disease in cultured common carp *Cyprinus carpio* in Japan in early November 2003. At that time, the Prefectural Fisheries Experimental Stations (PFESs), which belong to local government, lacked sufficient equipment and skill required to conduct a polymerase chain reaction (PCR) tests for KHV detection. Therefore, at first, most PCR tests for KHV were carried out at the National Research Institute of Aquaculture (NRIA), Fisheries Research Agency (FRA). The NRIA gave a short training course of PCR for the research staff of PFESs in mid-November. According to the established guideline for the KHV disease, the PFESs now conduct epizootic and routine clinical examinations on diseased fish followed by PCR test to detect viral DNA in the tissues. When there is a positive reaction with the PCR test, the sample is sent to the NRIA for further confirmation by PCR. Virus isolation on the KF-1 cell line is also tried, but confirmatory diagnosis is based on the results of PCR tests. By the end of 2003, the NRIA has accepted 529 individuals of 87 cases to be diagnosed and KHV-infected carp have been found in 23 out of 47 prefectures.

Key words:diagnosis, koi herpesvirus, KHV, specific disease, common carp, *Cyprinus carpio*

Diagnosis System for Exotic Diseases and Koi Herpesvirus

Some diseases are designated as “Specific Disease” in Japanese law. These are principally exotic diseases that have the potential to devastate the aquaculture industry in Japan such as spring viremia of carp (SVC) and viral hemorrhagic septicemia (VHS) of salmonid fish. For such diseases, protective guidelines have been established. The guidelines provide etiological information, diagnostic procedures, descriptions of the symptoms and other important characteristics of the disease. Laboratory diagnosis of the

disease should be conducted in accordance with these guidelines.

A newly isolated herpes virus, designated as koi herpesvirus (KHV), was first reported as a causative pathogen of mass mortality among common and ornamental (koi) carp *Cyprinus carpio* cultured in Israel and the USA in 1998 (Hedrick *et al.*, 2000). A similar virus was also isolated after massive mortality of carp in Germany (Neukirch and Kunz, 2001) and Israel (Perelberg *et al.*, 2003), and the virus, isolated in Israel, was identified as carp nephritis and gill necrosis virus (CNGV) based on the histopathological results (Ronen *et al.*, 2003).

Subsequently, this viral infection has been observed in western Europe since 2000*¹, Indonesia in the spring of 2002*² and Taiwan in the fall of 2002 (Tu *et al.*, 2004), revealing that this disease is rapidly spreading worldwide in carp-trading countries.

In Japan, there had been no such mass mortality in carp, and KHV had not been detected by a survey conducted in Niigata

Prefecture (Amita *et al.*, 2002). It has been shown that KHV is highly contagious and virulent in juvenile and adult carp (Hedrick *et al.*, 2000; Perelberg *et al.*, 2003).

Therefore, KHV infection was designated as a “Specific Disease” by a Japanese law amended on June 30, 2003, and an inspection procedure was established as a part of the guidelines (Fig. 1).

*¹: Le Deuff, R.-M., K. Way, L. Ecclestone, P. F. Dixon, A. M. Betts, D. M. Stone, O. Gilad, and R. P. Hedrick (2001): Abstract of the 10th International Conference of the European Association of Fish Pathologists, p 57.

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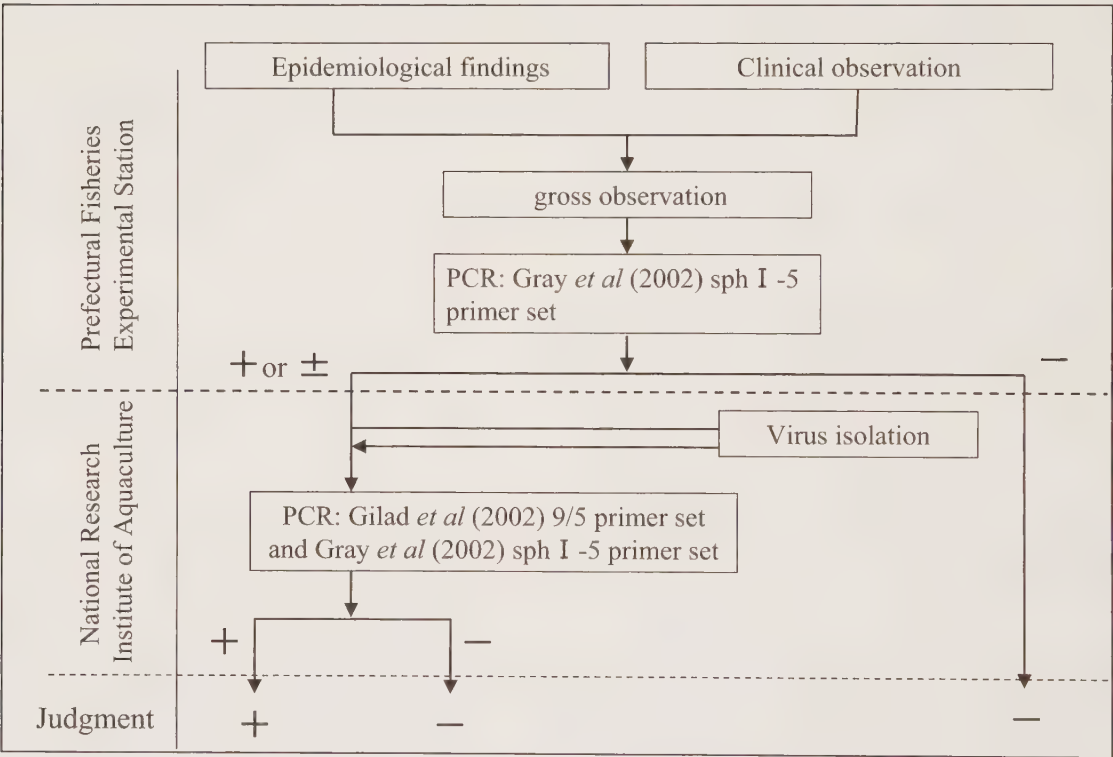


Fig. 1. Inspection procedure for KHV according to the Japanese guidelines.

According to the procedure, the Prefectural Fisheries Experimental Stations (PFESs), which belong to local government, first conduct epizootic and routine clinical examination on diseased fish. The most important epizootiological aspect of the KHV disease is that it only affects carp *Cyprinus carpio* and occurs apparently only in a limited range of temperature from 18 to 28°C (Hedrick *et al.*, 2000; Gilad *et al.*, 2003). Therefore, the water temperature and affected species of fish should be determined during a field examination.

Few external signs are usually visible, but pale and necrotic gills are frequently evident. *Flavobacterium columnare* (= *Flexibacter columnaris*) infection and some protozoan parasites, such as *Chilodonella* and *Trichodina*, are sometimes found on necrotic gill lesions, easily leading to misdiagnoses.

If any doubt remains as to the presence of KHV, a polymerase chain reaction (PCR) test can be used to detect KHV DNA in the tissues of fish. The PCR method described by Gray *et al.* (2002) was adopted in the inspection procedure as the primary examination to be conducted by the PFESs.

When the PCR test was positive for KHV, the sample is sent to the National Research Institute of Aquaculture (NRIA), Fisheries Research Agency (FRA), for further examination by the PCR methods of both Gilad *et al.* (2002) and Gray *et al.* (2002) for confirmation. Virus isolation on the KF-1 cell line is also attempted using the KF-1 cell line (Hedrick *et al.*, 2000). Because of the difficulty of isolating the virus in the cell line, the results of the isolation trial is treated as supplementary data, and confirmation of KHV is solely based on the results of the PCR examinations.

Occurrence of KHVD in Japan and Practical Diagnosis of the Disease

The mortality among common carp cultured in net pens at Lake Kasumigaura increased since the beginning of October 2003, when the water temperature of the lake was 16-18°C. The fish swam lethargically near the water surface. There were no marked external signs in the most of the affected fish, but a whitish mucous-like substance on the body surface, redness of the fin and body, fin rot, and discoloration of the gill with some necrosis were sometimes observed.

The mortality was over 60% in the most severe cases, especially in larger carp over 2 years old. The losses of the cultured carp were estimated to be 660 tons by early November and reached approximately 1,200 tons by mid-November, which is approximately 1/4 of the annual production of the lake.

External parasites, such as *Chilodonella*, *Trichodina*, and *Gyrodactylus*, were sometimes seen on the necrotic gill of the affected fish. Marked histopathological changes were observed in the gill of the diseased carp (Fig. 2).

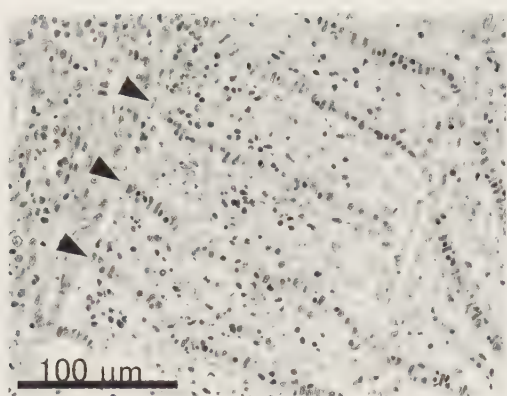


Fig. 2. A tissue section of the gill from affected common carp cultured at Lake Kasumigaura. Arrowheads indicate secondary lamellae, H&E stain.

The secondary lamellae were often fused with hyperplastic branchial epithelium where cell necrosis or the infiltration of lymphocytes

were often observed. Congestion and hemorrhage were sometimes observed. In some cases, the branchial tissues were severely degraded and numerous bacteria were seen in those lesions. These histopathological changes are similar to those of the previous reports (Hedrick *et al.*, 2000; Tu *et al.*, 2004). Unlike previous report (Hedrick *et al.*, 2000), nuclear changes characterized by hypertrophy and margination of chromatin were rarely observed. No bacteria was dominantly isolated from the kidney of affected fish were observed on trypticase soy agar.

The PCR test for KHV revealed specific bands amplified by the methods of Gray *et al.* (2002) and Gilad *et al.* (2002) (Fig. 3).

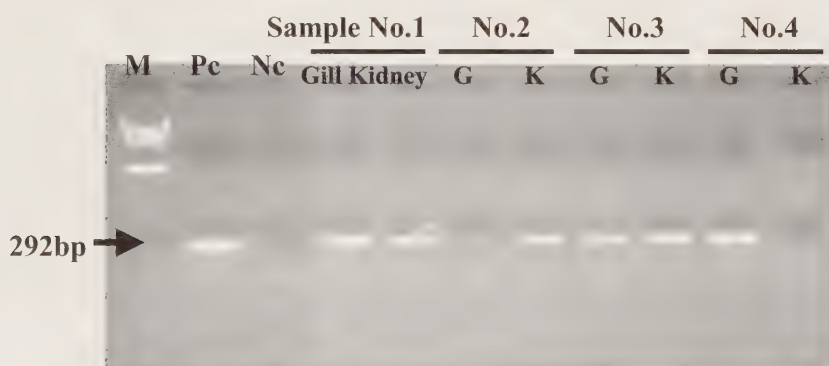


Fig. 3. Gel electrophoresis of the products (292bp) amplified with the primer set sph I-5 of Gray *et al.* (2002) from the extracted sample of gill and kidney of affected fish cultured in Lake Kasumigaura. M: marker, Pc: positive control, Nc: negative control. 1% agarose gel stained with ethidium bromide.

The sequence of the amplicon by the to the sequence deposited in the GenBank at accession no. AY568951, and that by the primer set of Gilad *et al.* showed 99% matching to AF411803.

The Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan officially announced the first case of KHV in Japan on November 2, 2003. At that time, the PFESs did not have sufficient equipment and technology for conducting PCR tests for

primer set of Gray *et al.* was identical KHV detection. Therefore, at first, most of the PCR tests for KHV were carried out at the NRA.

The NRA gave a short training course of PCR for KHV for the research staff of PFESs in mid-November. PCR inspection for KHV was then established in Japan in accordance with the procedure in the guidelines.

Evidence of the Presence of KHV before the Outbreak at the Lake Kasumigaura

Independently of the Lake Kasumigaura outbreak, a massive loss in excess of 10,000 carp occurred in some rivers and a lake in Okayama Prefecture in late May to mid-July 2003. However, in November 2003, the NRIA detected KHV DNA by PCR in samples of diseased fish stored in a freezer. This demonstrates that KHV had been introduced into Japan by May 2003, well before the Lake Kasumigaura outbreak.

The Spread of KHV in the Fall of 2003

The KHV-infected common carp cultured in Lake Kasumigaura had been transferred to the other areas in Japan before the first detection of the KHV, and, therefore, the virus had spread as a result. In some facilities, mortalities of carp with KHV were reported. However, in many cases, KHV was detected in carp when there was no mortality in the facility. This could be attributed to the fact that the water temperature was gradually decreasing at the time. By the end of 2003, the NRIA has diagnosed 529 individuals of 87 cases, and KHV had been found in 23 out of 47 prefectures in Japan. However, half of the cases had no obvious relations with the Lake Kasumigaura.

Acknowledgment

We are grateful to Dr. Ronald P. Hedrick of the University of California, Davis, USA, for his invaluable suggestions, which led to the establishment of the guidelines for KHV infection in Japan, and for providing a cell line and virus isolate. We also thank the Ibaraki Prefectural Experimental Station and the Okayama Prefectural Experimental Station for providing sample fish, and also thank the staff members of the Aquatic Animal Health Division of the National Research Institute of Aquaculture for diagnosing the KHV.

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The Status of Koi Herpesvirus Disease Outbreaks in Lake Kasumigaura and Kitaura

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Abstract

Lake Kasumigaura and Kitaura in Ibaraki Prefecture is the second largest lake in Japan. In the lake, common fisheries such as trawling and set net as well as net cage culture are practiced. Net cage culture produces about 5,000 tons of edible common carp (*Cyprinus carpio*) per year, which is one half of the total production in Japan. At the beginning of October 2003, deaths of unknown origin of common carp in net cages occurred in Lake Kasumigaura. After inquiry of the cause, PCR testing detected DNA of the Koi herpesvirus (KHV) in the affected fish and natural carp captured in Lake Kitaura, confirming the occurrence of the Koi herpesvirus disease (KHVD). We conducted an interview survey on aquaculturists regarding the date, quantity, and circumstance of death of cultured carp in Lake Kasumigaura and Kitaura, and investigated the circumstances of death in net cage farms. Another interview survey was also conducted on other fishermen regarding the occurrence of abnormality in natural carp, and both cultured and natural carps were sampled for PCR testing. The death of cultured carp in Lake Kasumigaura was generated in two areas dense with net cage farms, and then was likely expanded. The cumulative amount of death according to the interview surveys increased rapidly: 200-300 tons in the middle of October, 660 tons at the end of the same month, and 1,200 tons at the beginning of November. The damage amount reached one quarter that of the annual common carp products. Since the KHVD is one of the designated diseases under the Law to Ensure Sustainable Aquaculture Production, to prevent the spread of this disease according to this law, Ibaraki Prefecture requested aquaculturists of Lake Kasumigaura and Kitaura to exercise restraint on the transfer of cultured carp in net cages on November 2, and issued orders prohibiting transfer on November 12 and implementing incineration and landfill disposal on December 21. The incineration disposal commenced from January 20. This report aims to shed light on the state of mass cultured carp kills in Lake Kasumigaura and Kitaura, caused by this KHV, and the state of outbreaks of abnormal fish among cultured and natural carp.

Key words: koi herpesvirus, KHV, common carp, *Cyprinus carpio*, Lake Kasumigaura and Kitaura

Introduction

Lake Kasumigaura and Lake Kitaura in Ibaraki Prefecture constitute the second largest lake in Japan, with the combined area of 220 km², 56 feeder rivers, 2,160km² of basin area and 44 municipalities in the basin. With the mean depth and storage volume

being four meters and approximately 850 million tons, respectively, the water is used for industrial and agricultural purposes. In the lake (i.e. Kasumigaura, including Lake Kitaura), general fishery activities, such as trawl and set net fishery, and carp aquaculture are under way. The fish catch in the lake was 17,000 tons in 1978, but has declined and

recently has hovered around 2,000 tons. With carp one of the major fish species subject to capture, the annual catch is between 200 and 300 tons despite a decline in overall catches.

Carp aquaculture in net cages in the lake was launched in the 1960s and thrived due to the use of synthetic fiber, formula feed and automatic feeders, etc. At its peak, approximately 9,000 tons of carp were produced annually, and even now, approximately 5,000 tons per annum, which accounts for half the edible carp in Japan, are shipped to markets nationwide.

In early October 2003, edible carp cultured at net cages in Lake Kasumigaura died from an unknown cause and the deaths spread over the mid October. At that time, various possible causes were discussed, including indigestion caused by excessive feeding at low water temperatures, as well as oxygen deficiency and multiplication of *Skeletonema potamos* which occurred simultaneously. We conducted bacteria tests and bioassays, but stopped short of pinpointing the causes. The authors therefore requested the Training Center of Aquatic Animal Health Division (AAHD), National Research Institute of Aquaculture (NRIA), Fisheries Research Agency (FRA), for the diagnosis to identify the causes of death. Through the PCR test on October 31, KHV DNA was detected in both cultured carp in Lake Kasumigaura and natural carp in Lake Kitaura. As a consequence, it confirmed that the outbreak of the KHVD for the first time in Japan.

This report aims to shed light on the state of mass cultured carp which were killed by the cause of KHV in the lake, and the state of outbreaks of abnormal fish among cultured and natural carps.

Materials and Methods

Interview surveys

Interview surveys, including questions about the period when cultured carp died, the number of such deaths and the conditions at the time of death in the lake were conducted with individuals engaged in the aquaculture business. At the same time, conditions at the time of death at fishing grounds with net cages were also investigated. Interview surveys on the state of abnormal natural fish occurrences were also carried out with general fishery operators and the PCR test was performed on cultured and natural captured carps.

Toxicity test

Water was sampled at five locations in the lake, and concentrated solutions (5 x and 10 x) were prepared using the freeze concentration equipment.

Himedaka (orange-red variety of *Oryzias latipes*) and *Paratyia compressa improvisa* were used as test materials and observed for 96 hours.

Results and Discussion

Fig. 1 indicates the location of fishing grounds with net cages in the lake. Net cages crisscross in the lake and the total number of such cages is approximately 2,442: 2,148 cages in Lake Kasumigaura and 294 in Lake Kitaura. 58 aquaculture operators produce carp and other fishes by the net cage culture. Kasumigaura and Tamatsukuri towns have numerous farms and a total of 1,800 net cages are in use in towns. Net cages used for carp culture are 25 m² in area and approximately 2 m in water depth. Production is highly efficient since the baseline average production is 1.5 tons per net cage.

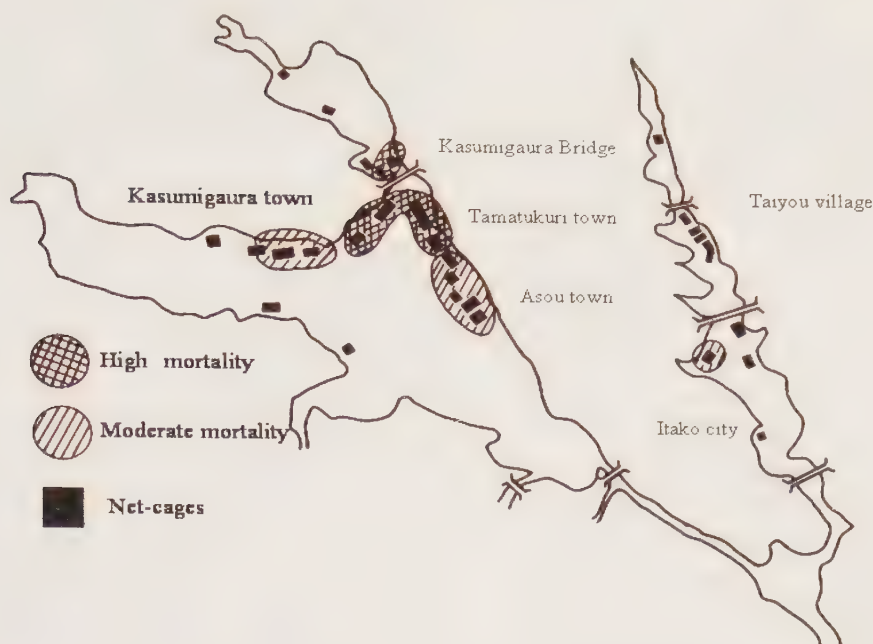


Fig. 1. Distribution of net-cages and KHVD damages in the lake.

Fig. 2 describes carp diagnosed with KHVD. Although diseased carp showed a variety of external symptoms, many of them suffered from albinism or friction on the surface or fins, and some appeared entirely red due to rube faction. Sunken eyes, anemia, hemostasis and discoloration of gills or decomposition of gills were identified.



Fig. 2. Symptoms of diseased fish. Symptoms of affected fish included sunken eyes, whitening of the body surface, and branchial anemia, congestion, and decomposition.

Comparison of disease symptoms between cultured carp and natural carp that tested positive to the PCR test appeared to indicate more advanced symptoms in natural

carp. Natural carp had significant hollowness of the eyeballs, they were thin all over, rubefaction was frequently detected on the surface of their body and their body surface felt rough due to deficient mucus.

The interview surveys of aquaculture operators on the behavior and conditions at the time of death, among other conditions, carp in net cages revealed the following trends:

- The number of deaths was high in net cages with high lipid content and large amounts of feed. Deaths also occurred, however, where no feed was given.
- In terms of body size, the number of deaths tended to be higher among large-sized carp compared with yearlings, and many aquaculture operators indicated that the number of yearling deaths was small.
- On the other hand, comparison between edible carp and red koi carp revealed that red koi carp began dying earlier.
- With regard to conditions at the time of death, there were cases in which healthy carp fed next to net cages where deaths occurred, cases where breeding groups of healthy carp that were fed until the day before deaths

occurred began to die from the following day and cases where some populations were observed swimming lethargically in the surface layer prior to death.

Since carp kills coincided with the formation of "red water" due to the multiplication of *Skeletonema potamos*, this was suspected as the cause of the deaths. Some individuals believed the deaths were caused by poisoning due to water quality deterioration or indigestion because of excessive feeding at low water temperatures. Since anoxia, pH, low-quality formula feed and various other possibilities were cited as probable causes, a bioassay was performed. No deaths occurred after 96 hours, which led to the conclusion that the carp kills were not caused by acute toxicity.

Taking into account the possibility of bacterial diseases, bacteria tests were conducted, however, no bacteria were separated. The possibilities of indigestion and inferior feed from specific manufacturers were precluded because unfeeding carp also died and deaths of cultured carp were not reported in the Kitaura district during this period.

Since a myriad of test results failed to identify the causes, we requested the NRIA on October 28 to identify the causes of the deaths using specimens submitted by us. As a result of the PCR test, KHV DNA was detected in sick cultured carp in Lake Kasumigaura and natural carp in Lake Kitaura on October 31, and an outbreak of KHVD was suspected.

On November 1, the Ministry of Agriculture, Forestry and Fisheries (MAFF) and the NRIA conducted field surveys and concluded that the possibility of KHVD was high. The Ibaraki Prefectural Government held a "Crisis-Response Meeting for Cultured Carp Deaths" on November 2 and called on aquaculture operators to cooperate in voluntary restraint of shipments.

In 2003, water temperatures of Lake Kasumigaura were lower than average during summer, then significantly fluctuated and dropped suddenly in October. The temperature in early October, when carp kills began, was 18°C and decreased to 14-15°C from late October to early November. It further declined to 12-14°C from mid to late November, when deaths had mostly ceased. Deaths ended earlier in aquaculture farms where they began earlier while deaths tended to come to an end later in aquaculture farms where the outbreak occurred later.

An overview of damages in the lake is described in Fig. 1. It appears from the interview survey results that deaths of cultured carp in Lake Kasumigaura began early in Kasumigaura and Tamatsukuri towns, two districts that have numerous fishing grounds with net cages. These two districts also suffered significant damage. Deaths began in fishing grounds with net cages located far away from these two districts in late October. Although the accumulated number of deaths was estimated to be 200 to 300 tons in mid-October, the said number rapidly increased over a short period of time and the affected area also spread in Kasumigaura district: 660 tons in late October and 1,200 (1,124) tons from November 5 to 7, when on-site inspections were conducted. The extent of the damage reached a quarter of the annual production. On-site inspections also confirmed that carp had died in 77% of net cages in the lake. Due partly to the fact that production volume was large in Lake Kasumigaura; deaths were confirmed in 1,828 net cages among 2,148 in Lake Kasumigaura. The quantity of deaths in Lake Kitaura at that time was still low at approximately two tons.

Fig. 3 is a pattern diagram in which the period of death occurrences in two districts in Lake Kasumigaura, where deaths began early, was classified every five days from October 1.

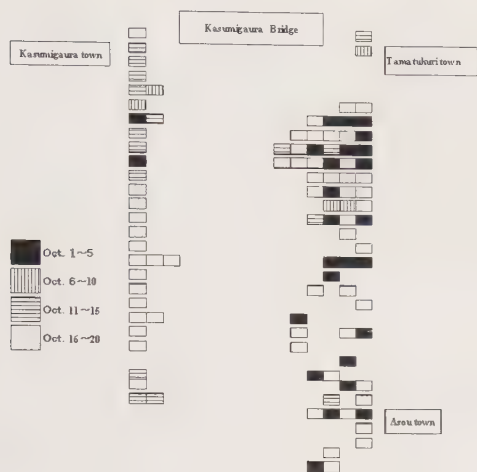


Fig. 3. A pattern diagram in which the period of death occurrences in two districts in Lake Kasumigaura.

This diagram implies that deaths began at various fishing grounds almost simultaneously, rather than as result of the outbreak of disease spreading in concentric circles from a single net cage. Since it was also conceivable for cultured carp to become infected from natural carp, we investigated the relationship between the period when carp deaths broke out in an aquaculture farm and when natural carp caught elsewhere were placed in the net cage of the farm. Nevertheless, the relationship between the breeding of natural carp and mortality outbreaks was not clear. Fig. 4 shows the distribution of cultured carp and natural carp which tested positive to the PCR test.

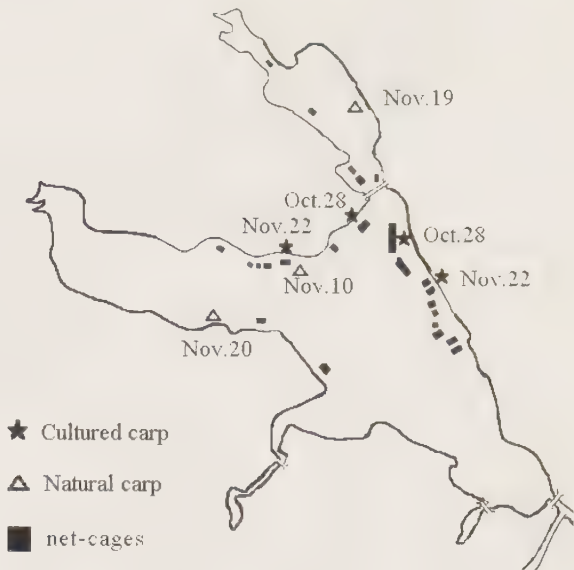


Fig. 4. Detection of PCR positive carp in Lake Kasumigaura.

Interview surveys on the abnormality of natural carp in various places were conducted with general fishery operators from October 23. There was no information on abnormal carp in Lake Kasumigaura until October 31. As a result of the PCR test, KHV DNA was detected in carp caught on November 10, 19 and 20.

Fig. 5 indicates the state of abnormal carp appearances in Lake Kitaura.



Fig. 5. Detection of PCR positive carp and abnormal natural carp in Lake Kitaura.

- 1) Abnormal natural carp since Oct. 29
- 2) Abnormal natural carp since Oct. 20
- 3) Mortality occurred since Oct. 12
- 4) Abnormal natural carp since last Sept. PCR positive natural carp on Oct. 28
- 5) Low mortality. PCR positive cultured carp on Nov. 22
- 6) Mortality occurred since Nov. 9
PCR positive cultured carp on Nov. 15
- 7) Normal natural carp until Oct. 27
- 8) Abnormal natural carp since Oct. 20

In the Kitaura district, interview surveys were conducted after cultured carp kills occurred. As a result, it was found that abnormal natural carp, which were extremely lean and suffered rubefaction on the surface, had been caught in Itako city in the downstream basin from around late September, i.e. prior to the outbreak of cultured carp deaths. Natural carp caught at identical places on October 28 tested positive to the PCR test.

Subsequently, deaths of cultured carp began in Aso town around October 12, followed by the catch of carp with rubefaction on the surface at two locations in the upstream and downstream basins where the populations that tested positive to the PCR

test were initially caught on October 20. Information came to hand on October 29 that abnormal natural carp had been caught a long way upstream. Cultured carp began dying in the midstream basin on November 9, and most cultured carp that were sampled on November 15 tested positive to the PCR test. Although there were few deaths among cultured carp in the uppermost stream of Lake Kitaura, samples collected on November 22 showed positive reactions to the PCR test.

In the Kitaura district, information that abnormal natural carp had been caught in the downstream areas was first made available.

KHVD was confirmed in natural carp first, and carp that ordinary fishery operators found to be abnormal in appearance were subsequently confirmed in the upstream areas. They were followed by the onset of cultured carp kills and the appearance of populations the tested positive to the PCR test. These were characteristics of the mass carp kills.

In addition to carp, channel catfish, *Ictalurus punctatus*, silver carp, *Hypophthalmichthys molitrix*, crucian carp, *Carassius auratus*, etc. are cultured in the net cage aquaculture. Nevertheless, deaths of the aforementioned fish species were not observed while large quantities of carp died. Aiming to prevent damage from harmful rumors, the PCR test was carried out on lake smelts, (*Hypomesus nipponensis*), ice fish (*Salangichthys microdon*), gobies (*Tridentiger kuroiwaie brevispinis*) and freshwater prawns (*Macrobrachium nipponense*), then all results were negative.

As countermeasures for the spread of the disease, the Ibaraki Prefectural Government called on aquaculture operators for voluntary restraint on shipments based on the Law on Ensuring Sustainable Aquaculture Production on November 2. From November 5 to 7, the prefectural government conducted on-site inspections of all net cages where carp deaths were confirmed and issued an injunction on November 11 against transferring carp in net cages where deaths were confirmed. On

November 30, another injunction was issued against transferring carp from all net cages. On December 20, disposal through incineration and burial was ordered and incineration was initiated from January 20, 2004.

Nevertheless, precritical cultured carp with no symptoms had already been shipped to carp-consuming areas nationwide before the detection of the KHV DNA by PCR test. Because live fish were shipped according to the general pattern of carp distribution; The lake produced half the cultured edible carp in the nation; it coincided with the shipping period; and the latent period of KHVD was long, i.e. for two to three weeks. The first outbreak of KHVD was confirmed at the lake, and outbreaks of KHVD were then confirmed throughout the nation. KHVD outbreaks included a case in which the phenomenon of mass carp kills was recognized in natural waters in Okayama Prefecture prior to the outbreak of KHVD at the lake. The aforementioned mass carp kills were handled as deaths from other causes and KHVD was confirmed in frozen specimens at a later date. And the infection routes were unknown among half cases of 84 KHVD outbreaks in Japan until December (announcement of the MAFF of Japan). These cases highlighted the importance of preventing non-native diseases from entering Japan, the necessity of developing and disseminating diagnostic technology for new diseases and the significance of initial prevention of spread of such diseases. This survey on the state of KHVD outbreaks revealed that the mass deaths in Lake Kasumigaura began with simultaneous multiple deaths at all aquaculture farms in Kasumigaura and Tamatsukuri towns, rather than occurring at specific fishing grounds with net cages and spreading from there. Since abnormality of natural carp broke out in Lake Kitaura before deaths of cultured carp occurred, the possibility of natural carp getting involved in the expanded infection among cultured carp was also taken into consideration. On the

other hand, the outbreak of cultured carp deaths in Lake Kasumigaura and the confirmation of abnormal natural carp in Lake Kitaura were almost concurrent, suggesting the possibility that there might have been two routes of infection.

The outbreak of KHVD had never been reported in natural waters and it was confirmed in the lake for the first time. At small-sized aquaculture farms in closed waters, it is possible to eradicate pathogens by killing diseased fish and disinfecting aquaculture farms and to resume aquaculture easily. In open waters with an expansive catchments area, whose water is widely used for industrial and agricultural purposes, for example Lake Kasumigaura, pathogen eradication measures are not practical. Resumption of aquaculture business in the lake, where the production quantity of cultured carp accounts for 50% of the national total, however, urgently requires the development of vaccines, the breeding of disease-resistant fishes, among other measures, in addition to countermeasures for the spread of disease and virus eradication measures.

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We are grateful to the staff members of the AAHD, NRIA for diagnosis of KHVD and to the staff members to Ibaraki Prefecture Freshwater Fishery Research Institute (IPFFRI) and Kasumigaura-Kitaura Fishery Office for interview surveys.

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Preventive Measures against Koi Herpesvirus Disease in Fancy Carp in Niigata Prefecture

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Objectives

Following by the outbreaks of koi herpesvirus (KHV) in worldwide including Israel, many areas of Europe, the United States, and Indonesia, the Fisheries Division of the Niigata Prefectural Government started implementing a polymerase chain reaction (PCR) tests on the prefecture's carp in October of 2001.

The aim of the test was twofold--to prevent the entry of the KHV into the prefecture and to prevent the spread of the virus within and out of the prefecture, both domestic and abroad, if the virus is detected in carp in Niigata. Although there have been no reports of a KHV outbreak in Niigata's fancy carp, the prefecture's fancy carp industry has voluntarily suspended all of its shipments temporarily. This measure has been taken to prevent the possible transfer of the virus following the detection of KHV in common carp in Niigata on November of 2003 (Note: These KHV-affected fish, originally imported from Ibaraki prefecture, were obtained from an entertainment fishing pond and a breeder of common carp in Niigata prefecture.).

Method

A polymerase chain reaction (PCR) tests were conducted in Niigata prefecture during the following three periods: from October to November of 2001 on fancy

carp owned by twenty carp breeders, in December of 2002 on fancy carp owned by thirty carp breeders, and from July to August of 2003 on fancy carp owned by 114 breeders. (Note: Many of the breeders have participated in the PCR tests in multiple years.)

The procedures of PCR-test were as follows (Fig. 1): 1) To avoid a great financial loss, sample fish were selected only for young fancy carps less than one-year old which mean valueless in terms of unable to reproductive activities. 2) These young carps were kept in tank during 3 weeks under maintaining condition of water-temperature from 20°C to 25°C, together with adult ones that would be shipped for commercial purposes. 3) Sample fish for examined were maintained by designated staff members who conduct PCR tests under the supervision of the Niigata Prefectural Inland-water Fisheries Experimental Station. 4) Afterwards tissues from the gill, spleen, kidney, and heart (sometimes just a sample from the gill tissue) were taken from two to five of each sample fish. 5) These tissues were homogenized to extract DNA, which was obtained by using a DNA extraction kit. 6) Each sample was mixed with an agent that contained types of primers such as 9/5 and SphI-5. 7) After this stage, the samples received an agarose gel electrophoresis treatment to examine the DNA fragments. and 8) A KHV DNA sample, which Niigata had received from Dr. R. P. Hedrick, was used to compare the

results of the samples taken in Niigata.

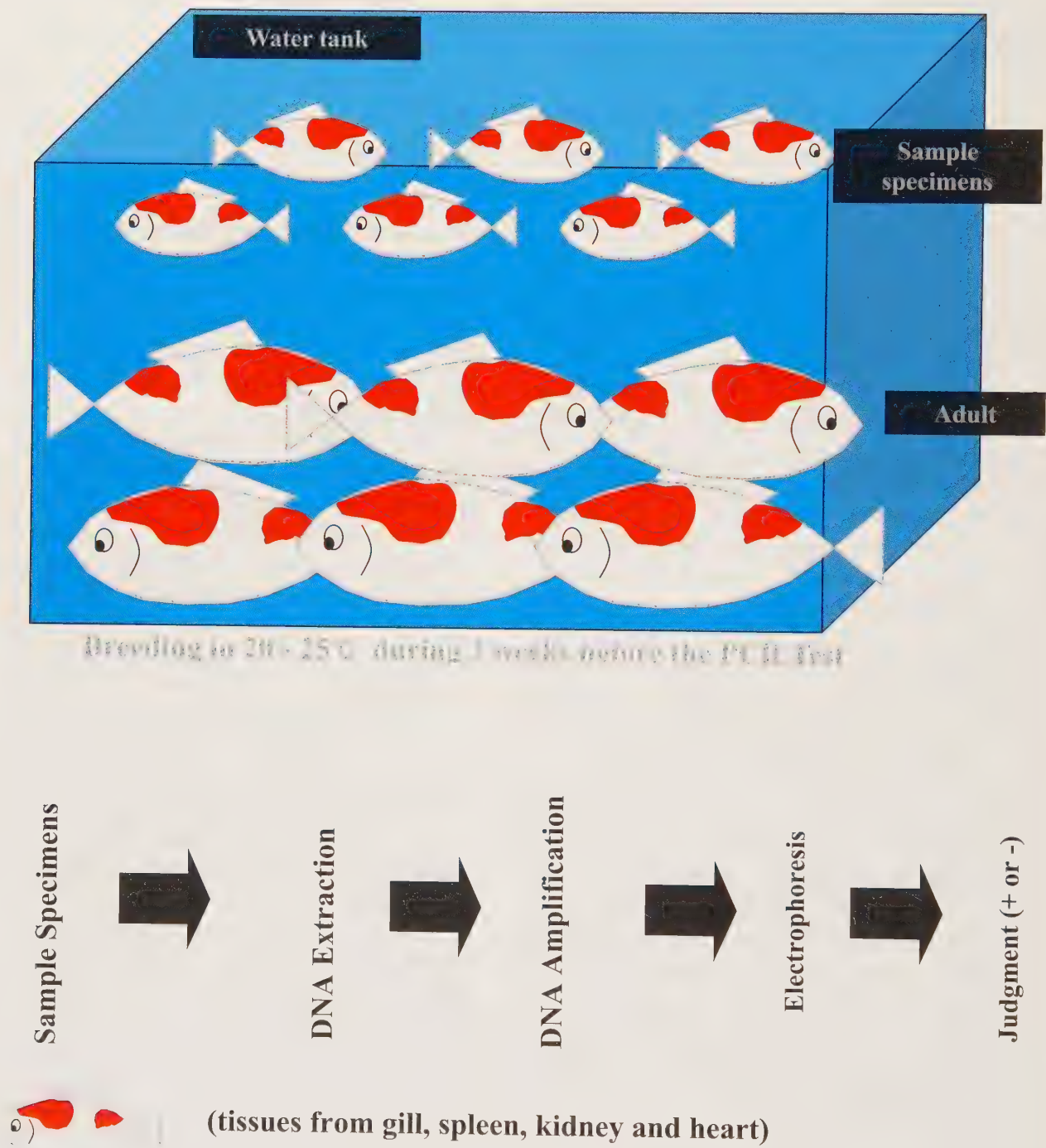


Fig. 1. PCR-Test Method.

Results

All the fish that were examined in the PCR tests during the previously specified periods tested negative. During these test periods also, Niigata provided carp business owners in the region with meetings and lectures about KHV to enable the breeders to be able to detect KHV-affected carp at an early stage of the disease and thus minimize the risk of spreading the virus.

Currently PCR tests are being conducted on the largest scale ever in

Niigata with the participation of 150 carp breeders, each of which presented tissue samples from thirty-one fish from their farm. Originally, the voluntary policy to temporary suspend ornamental carp shipments prohibited any shipments of fancy carp until the test results of the fish from all the breeders concerned were confirmed; however, the voluntary policy to temporary suspend fancy carp shipments has been revised to allow overseas shipments of fish upon confirmation of favorable test results of the fish from each individual breeder (Table 1).

Table 1. Results of PCR-testing in Niigata Prefecture.

Test-Date	Oct.-Nov., 2001	Nov.-Dec., 2002
Method	PCR (9/5 Primer)	PCR (9/5 Primer)
DNA Extraction Kit (Agents)	ISOGEN	ISOGEN
A tissues (section) from	Gill, Brain, Spleen, Kidney	Gill, Brain, Spleen, Kidney
No. of samples examined	205 individuals (42 specimens)	420 inds (84 specimens)
No. of enterprise examined	20 enterprise	30 enterprise
Results of PCR-test	All negative (-)	All negative (-)

Upon Niigata Prefectural Government’s request, the carp industry in Niigata is creating a KHV prevention policy that instructs carp breeders how to avoid exposing fish to the virus. Under this policy, any fish that is shipped from another breeder will have to be isolated from other fish in a low temperature water tank for three weeks during which time the breeder will check to see whether or not

the fish is infected with the virus. It also advises the breeder to avoid using river water when maintaining the fish and to sanitize all equipment that comes in contact with fish water or the fish themselves, including staff’s boots and gloves. Staff members are also required to wash their hands at their facility and to keep a detailed journal of the conditions of their fish.

Further Researches for the Control of KHVD in Japan

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In the fall of 2003, the first outbreak of Koi Herpes Viral disease occurred in Lake Kasumigaura in Japan, and the disease spread rapidly over the country. The disease has caused massive mortality in both cultured and wild carp. As the water temperature has decreased in winter months, the disease seems to have been suppressed now, but people fear that the disease will reappear in this coming spring through summer. Prevention of KHV outbreak in cultured carp and control of the disease in wild waters are strongly required.

To achieve these goals, Fisheries Research Agency is planning a research project in collaboration with both academic and private sectors. There are three major areas of research or development in the project. In the first area, the basic pathogenesis of the disease and molecular genetics of KHV will be studied. Since the mechanisms or steps whereby KHV brings about disease in carp are largely unknown, these will be studied using histopathological techniques.

Epizootiological study including molecular genetics of the virus will also be conducted, so that the spreading route of the

disease in Japan will be clarified, at least partly.

Second, new diagnosis or detection methods for KHV will be developed. Currently, diagnosis of the disease is exclusively carried out by PCR. However, this method requires a thermal cycler, and there is a concern that the present PCR methods for KHV may not be sensitive enough to detect the virus at the latent stage of the disease. On the other hand, PCR has a possibility of yielding false positive results by contamination of a minute amount of viral genes. Hence, techniques with which we can overcome these problems will be developed, as well as improvement of the present PCR techniques.

Third, preventive measures for KHV should be developed. Susceptibility of KHV to various chemicals and physical treatments will be tested to develop effective methods of disinfection of fish farming equipment and rearing water. Development of inactivated vaccine and effective vaccination techniques will also be tried. Furthermore, a procedure will be developed to cure the diseased fish by raising water temperature.

Proposed Activities for Koi Herpesvirus Disease at the SEAFDEC Aquaculture Department

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Abstract

The Southeast Asian Fisheries Development Center (SEAFDEC) is a regional treaty organization with 11 member countries. This was established in 1967 to promote fisheries development in Southeast Asia. As one of four SEAFDEC departments, the Aquaculture Department based in Tigbauan, Iloilo, Philippines, has conducted activities for aquaculture research and development in the region. Since 2000, the Regional Fish Disease Project has been implemented at the SEAFDEC Aquaculture Department through the Government of Japan Trust Fund. Under this project, research studies were conducted on various aspects of viral, bacterial and parasitic diseases of fishes and shrimps. In East Asia, koi herpesvirus (KHV) disease initially occurred in Indonesia and Taiwan in 2002. KHV infection was also found in Japan in 2003. This disease had a serious, devastating impact on common carp and koi (*Cyprinus carpio*) production in Indonesia and common carp production in Japan. Common carp is an important food resource in the rural areas of the region, while koi is internationally traded as ornamental fish among Southeast Asian countries. Under these situations, the Regional Fish Disease Project identified KHV as a serious, transboundary pathogen in the region and decided to work on KHV disease at the SEAFDEC Aquaculture Department in coordination with the SEAFDEC member countries to prevent the spread of KHV in the region. The planned research includes survey of the distribution of KHV in the region, standardization of the PCR (polymerase chain reaction) detection method, characterization of the virus isolated from the region, mode of transmission of KHV, and pathophysiology of KHV-infected fish. To support establishment of the fish disease quarantine and surveillance in Southeast Asia, the Regional Fish Disease Project has, since 2002, annually conducted a hands-on training at the SEAFDEC Aquaculture Department on viral diseases of fishes and shrimps for scientists and technical staff from the SEAFDEC member countries. The trainees are expected to play key roles in the diagnosis, prompt information exchange, and surveillance of fish diseases, including KHV disease, in their respective countries. The Regional Fish Disease Project organized two meetings in March 2004 and will convene another meeting in June 2004: Pre-KHVD Symposium Meeting, International Symposium on Koi Herpesvirus Disease, and Meeting on Current Status of Transboundary Fish Diseases in Southeast Asia: Occurrence, Surveillance, Research and Training.

Key words: koi herpesvirus disease, KHV, common carp, koi, *Cyprinus carpio*, Southeast Asian Fisheries Development Center, Southeast Asia, Regional Fish Disease Project, proposed activities

What is the SEAFDEC Aquaculture Department ?

The Southeast Asian Fisheries Development Center (SEAFDEC) is a regional treaty organization established in

1967 to promote fisheries development in Southeast Asia. Its member countries are Japan, Malaysia, Philippines, Singapore, Thailand, Brunei Darussalam, Socialist Republic of Vietnam, Myanmar, Indonesia, Cambodia, and Lao PDR. The Chief

administrator of SEAFDEC is the Secretary-General whose office, the Secretariat, is based in Bangkok, Thailand.

Four departments were established to pursue the objectives of SEAFDEC: (1) the Training Department in Samut Prakan, Thailand, for marine capture fisheries training; (2) the Marine Fisheries Research Department in Singapore for fishery post-harvest technology; (3) the Marine Fishery Resources Development and Management Department in Kuala Terengganu, Malaysia, for the development and management of the marine fishery resources in the exclusive economic zones of the SEAFDEC member countries; and (4) the Aquaculture Department in Tigbauan, Iloilo, Philippines, for aquaculture research and development.

Since its establishment in 1973, the Aquaculture Department has been mandated to: (1) promote, undertake and coordinate aquaculture research relevant to Southeast Asia; (2) develop human resources for aquaculture development; and (3) disseminate and exchange information in aquaculture. The first mandate is the primary function of the Research Division, which undertakes research on commercially important species of fishes, crustaceans, molluscs, seaweeds, and other aquatic organisms. Research on disease diagnosis and health management of aquatic organisms is one of the main research activities at the department. Under the Research Division is the Fish Health Section with five scientists (4 with PhD and 1 with MS).

The Regional Fish Disease Project Supported by the Government of Japan Trust Fund

The “Regional Fish Disease Project” has been implemented at the SEAFDEC Aquaculture Department through the Government of Japan Trust Fund to address various fish disease problems and food safety

issue in Southeast Asia (Inui, 2002; Nagasawa, 2004). The first phase of the project entitled “Development of Fish Disease Inspection Methodologies for Artificially-Bred Seeds” started in 2000 and will end in 2004. It was initially planned to end in 2003 but it was extended to 2004 because of the urgent need to study KHV infection. After this first 5-year project, the second phase of the Regional Fish Disease Project has been proposed under the title of “Development of Fish Disease Surveillance System” for another 5 years from 2004 to 2008.

The Regional Fish Disease Project aims to: (1) enhance disease diagnosis and health management of aquatic animals in aquaculture in Southeast Asia; (2) promote the healthy and wholesome trading of aquaculture products in the region; and (3) develop a fish disease surveillance network in the region.

To achieve these objectives, the project conducted the following activities from 2000-2004:

1. Research to (1) develop standardized diagnostic methods for major diseases affecting economically important aquaculture species in the region; (2) develop effective prevention and control measures against microbial and parasitic diseases; (3) assess the pathogenesis of newly emerging diseases; and (4) develop monitoring methods for residual chemicals in aquaculture products.

2. Hands-on training to develop capability in aquatic animal health diagnosis and management of technical staff working at research centers and institutions in the region.

3. International meetings to (1) discuss the status of fish disease problems, available diagnostic methods, and prevention and control measures employed in the region; (2) discuss the results of research studies conducted under the project and those generated in other countries in the region; (3) identify and discuss aquatic animal disease issues to be solved for further sustainable aquaculture growth; and (4) discuss

collaboration with other international organizations such as the Office International des Epizooties (OIE).

4. Extension to disseminate research results and technology generated by the Project through (1) training courses on fish diagnosis and health management; (2) production of manuals; (3) publication of primary results in international scientific journals; and (4) presentation of results in international meetings.

Research is the main activity component of the Regional Fish Disease Project. Various aspects of viral, bacterial and parasitic diseases of fishes and shrimps have been studied. When the project started in 2000, research was undertaken only by scientists of the SEAFDEC Aquaculture Department. Subsequently scientists of research institutions under the Department of Fisheries, Thailand, and those of the Marine Fisheries Research Department of SEAFDEC in Singapore joined the project in 2001 and 2002, respectively. A total of 24 research studies were conducted from 2000 to 2003 in terms of (1) establishment and standardization of diagnostic methods, (2) biology and pathogenesis of disease pathogens, (3) disease prevention and control, and (4) establishment of evaluation methods for residual chemicals in aquaculture products. Detailed information on the 24 research studies is given as Appendix.

To coordinate and promote the project, two Japanese senior scientists (designated as "Fish Disease Expert") were dispatched to the SEAFDEC Aquaculture Department (Dr. Yasuo Inui from March 2000 to March 2003; Dr. Kazuya Nagasawa from April 2003 to date).

KHV is a New, Serious Threat to Aquaculture in Southeast Asia

In Southeast Asia, koi herpesvirus (KHV) infection was first detected in common carp and koi (*Cyprinus carpio*) cultured in Indonesia in March 2002 (Sunarto *et al.*,

2002 Sunarto *et al.*, 2005). There is fragmentary information that KHV is also present in Malaysia (see table 1 in Gilad *et al.*, 2003). In neighboring East Asia, KHV disease was found in pond-reared koi from Taiwan in December 2002 (Tu *et al.*, 2004) and in common carp cultured in Japan in November 2003 (Sano *et al.*, 2004, 2005). In Indonesia, there have been numerous cases of KHV-induced mass mortality of common carp and koi since March 2002. Losses were estimated to be more than 15 million US dollars as of December 2003 (Sunarto *et al.*, 2005).

Common carp is an important food resource in the rural areas of Southeast Asia and abundantly cultured especially in Indonesia. Koi, on the other hand, is internationally traded as ornamental fish among Southeast Asian countries. Considering its high virulence and devastating impact on the aquaculture sector, KHV is regarded as a new, serious threat to common carp and koi aquaculture in the region.

Proposed Activities for KHV Disease under the Regional Fish Disease Project

Under these situations, study leaders involved in the Regional Fish Disease Project assembled at the Annual Progress and Planning Meeting held in Iloilo City, Philippines on 2-3 December 2003 and discussed fish disease issues for 2004 and onward. From the discussion, the following viruses were identified as serious, transboundary pathogens that the Regional Fish Disease Project should tackle as targets for fish disease surveillance in Southeast Asia: for fishes, KHV, spring viremia of carp virus (SVCV) and grass carp (*Ctenopharyngodon idella*) hemorrhagic virus (GCHV), and for shrimps and prawn, white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) of black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) and extra

small virus (XSV) associated with white tail disease of the giant freshwater prawn (*Macrobrachium rosenbergii*). In particular, KHV was recognized as the pathogen that the project must combat urgently.

Based on the output of the meeting, the SEAFDEC Aquaculture Department made a plan to implement various activities for KHV infection through the Regional Fish Disease Project. Some research studies were planned for 2004 under the first phase of the project, while others were for 2004-2008 under the second phase. The SEAFDEC Aquaculture Department believes that the project should proceed efficiently in coordination with the SEAFDEC member countries. Important activities for KHV disease, some of which were initiated in the first half of 2004, are as follows:

1. Research

During the first phase of the project, planned research will involve the survey of the distribution of KHV in the region, standardization of the PCR (polymerase chain reaction) detection method, characterization of the virus isolated from the region, mode of transmission of KHV and pathophysiology of KHV-infected fish. These studies will be undertaken at the SEAFDEC Aquaculture Department.

During the second phase of the project, when new information on KHV infection becomes available in the SEAFDEC member countries, the SEAFDEC Aquaculture Department will dispatch its diagnosis team to the disease site in order to isolate the virus and diagnose the disease together with scientists of the country in question. Also, there will be a study to develop vaccines for KHV, using inactivated virus or recombinant viral envelope protein, at the Fish Health Research Laboratory in Jakarta, Indonesia.

In addition to these activities under the Regional Fish Disease Project, the SEAFDEC Aquaculture Department will join in 2004 a 3-year research project on KHV infection, which will be funded by the

Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan. The National Research Institute of Aquaculture (based in Nansei and Tamaki, Mie, Japan) leads the project. Comparison of characteristics of KHV isolates from Asian countries is a research subject to be tackled by the SEAFDEC Aquaculture Department.

2. Hands-on training

Since 2002, the SEAFDEC Aquaculture Department has annually conducted a hands-on training on viral diseases of fishes and shrimp for scientists and technical staff working at research centers and institutions in Southeast Asia. The training course aims to provide an executive training on the diagnosis of viral diseases to core persons from the SEAFDEC member countries. The trainees are expected to play key roles in the diagnosis, prompt information exchange, and surveillance of fish diseases as well as to serve as national trainers in their respective countries. For 2004, a special training course on KHV and some other important viral pathogens is planned for scientists from some of the SEAFDEC member countries.

3. International meetings

The Regional Fish Disease Project organized two meetings in March 2004 and will convene another meeting in June 2004: (1) Pre-KHVD Symposium Meeting; (2) International Symposium on Koi Herpesvirus Disease; and (3) Meeting on Current Status of Transboundary Fish Diseases in Southeast Asia: Occurrence, Surveillance, Research and Training.

The Pre-KHVD Symposium Meeting was organized by the project at the Conference Room of the National Research Institute of Fisheries Science (NRIFS), Fisheries Research Agency (FRA) in Yokohama, Japan, on 12 March 2004 as a satellite meeting of the International Symposium on Koi Herpesvirus Disease. Participants to this meeting were nine scientists from the SEAFDEC member countries (one

participant from each country: Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand, Vietnam) and three scientists from the SEAFDEC Aquaculture Department. The scientists from the member countries reported on the current status of KHV disease, fish disease quarantine and surveillance in their respective countries. Participation of 11 scientists from Southeast Asia (excluding Thailand) in the meeting was funded by the project.

The International Symposium on Koi Herpesvirus Disease was co-organized by FRA, SEAFDEC (through the Regional Fish Disease Project), MAFF, and OIE in Yokohama on 13 March 2004. This meeting was participated in by scientists from Japan, the United States, South Korea, China, Israel and the Netherlands as well as scientists from the SEAFDEC member countries and Aquaculture Department. After the keynote lecture by Dr. Ronald P. Hedrick of the University of California, Davis, a total of 15 papers were presented by invited speakers. Four of these invited speakers came from Southeast Asia: Drs. Agus Sunarto (Indonesia), Ling Kai Huat (Singapore), Somkiat Kanchanakhan (Thailand) and Kazuya Nagasawa (SEAFDEC Aquaculture Department). Information presented at the symposium varied from basic knowledge of KHV through epidemiology of KHV infection in Indonesia and Japan, and KHV vaccine development in Israel to fish disease quarantine in Singapore and Thailand. It was useful in understanding of various aspects of KHV infection including control and prevention.

The Meeting on Current Status of Transboundary Fish Diseases in Southeast Asia: Occurrence, Surveillance, Research and Training in Manila, Philippines, on 23-24 June 2004 is being organized by the project. The meeting aims to exchange the latest information on transboundary fish diseases and fish surveillance, research and training in Southeast Asian countries. The project will

fund 11 scientists from all SEAFDEC member countries, two invited speakers from Taiwan and Canada and 10 scientists from the SEAFDEC Aquaculture Department to participate in the meeting. Two scientists each from OIE in Tokyo and the Network of Aquaculture Centres in Asia-Pacific (NACA) in Bangkok will also attend the meeting. As transboundary fish and shrimp pathogens, KHV, WSSV and TSV will be highlighted. The meeting will consist of five discussion sessions: (1) KHV; (2) WSSV and TSV; (3) quarantine services of aquatic animal diseases; (4) surveillance, monitoring and diagnosis of aquatic animal diseases; (5) research and training on diseases of aquatic animals. For each session, at least one invited lecture will be given, followed by reports from 10 Southeast Asian countries. During the first session, the current status of KHV infection in Indonesia, Taiwan and Japan will be reported by Drs. Agus Sunarto, Chien Tu and Motohiko Sano, respectively. For the second to fifth sessions, invited papers will be presented individually by Drs. Leobert D. de la Peña (SEAFDEC Aquaculture Department), J. Richard Arthur (Canada), Yoshiyuki Oketani (OIE) and Michael J. Phillips (NACA). Through the country reports, detailed information on the current status of KHV, WSSV and TSV as well as fish disease quarantine, surveillance, monitoring, diagnosis, research and training in Southeast Asian countries will be assembled.

4. Extension

Research results will be published in international scientific journals, and standardized PCR diagnostic techniques will be disseminated through manuals and hands-on training. As the output of the Pre-KHVD Symposium Meeting, one report (Nagasawa and Lio-Po, 2004) is available at the SEAFDEC Aquaculture Department. FRA will publish the proceedings of the International Symposium on Koi Herpesvirus Disease in the Bulletin of the Fisheries

Research Agency, Supplement No. 2. By October 2004, the SEAFDEC Aquaculture Department will publish the proceedings of the Meeting on Current Status of Transboundary Fish Diseases in Southeast Asia: Occurrence, Surveillance, Research and Training (Lavilla-Pitogo and Nagasawa, 2004).

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Appendix

Under the Regional Fish Disease Project, the following research studies were conducted during first phase of the project from 2000 to 2003 in four categories:

A. Establishment and Standardization of Diagnostic Methods

1. Standardization of diagnostic methods for viral diseases of shrimp (SEAFDEC Aquaculture Department [AQD], 2000)
2. Standardization of PCR technique as the detection method for WSSV infection in *Penaeus monodon* (SEAFDEC/AQD, 2001)
3. Development of shrimp cell culture *in vitro* (Marine Shrimp Research and Development Center [MSRDC], Thailand, 2001-2002)
4. Standardization of diagnostic methods for monodon baculovirus (MBV) and hepatopancreatic parvovirus (HPV): Establishment of monoclonal antibodies (MAbs) against MBV and HPV (SEAFDEC/AQD, 2001-2003)
5. Epizootiology of economically important viral diseases of wild *Penaeus monodon* (SEAFDEC/AQD, 2001-2003)
6. Viral diseases of cultured marine fishes in Southeast Asia
- 6-1. Detection and identification of viral pathogens in cultured marine finfish in the Philippines (SEAFDEC/AQD, 2000-2003)

- 6-2. A viral survey in diseased grouper in Thailand using virus isolation and polymerase chain reaction (PCR) technique (Aquatic Animal Health Research Institute [AAHRI], Thailand, 2001-2002)
- 6-3. Survey of iridoviral disease in freshwater fish in Thailand (AAHRI, Thailand, 2003)
7. Establishment of preventive measures against viral nervous necrosis (VNN) in fish broodstocks: (1) grouper, (2) milkfish, (3) red snapper, and (4) sea bass (SEAFDEC/AQD, 2001-2003)

B. Biology and Pathogenesis of Disease Pathogens

1. Parasitosis in marine and freshwater fish: diagnosis, pathology, prevention and control of infection
- 1-1. Screening of important parasites in economically important aquaculture fish (SEAFDEC/AQD, 2000-2003)
- 1-2. Biology and pathology of the gill monogenean parasitic to grouper (SEAFDEC/AQD, 2000-2003)
- 1-3. Leech infestation and its associated blood parasitic protozoans (SEAFDEC/AQD, 2000-2003)
- 1-4. Establishment/application of prevention and control methods against parasites (SEAFDEC/AQD, 2000-2003)
2. Study on parasites of grouper in Thailand (AAHRI, Thailand, 2001-2002)
3. Screening of important parasites of freshwater fish in Thailand and neighboring countries (AAHRI, Thailand, 2003)

C. Disease Prevention and Control

1. Use of bacteria as biological control agent against microbial diseases in shrimp (*Penaeus monodon*) and crab (*Scylla serrata*) hatcheries (SEAFDEC/AQD, 2000-2003)
2. Screening of probiotics as biocontrol/bioremediation in the rearing

- of *Penaeus monodon* I. Tank experiment (SEAFDEC/AQD, 2001-2003)
 3. Antibacterial metabolites in the microbial and phytoplankton flora of the "green water" cultured *Penaeus monodon* (SEAFDEC/AQD, 2000-2003)
 4. Investigation on the mechanism of the effect of tilapia culture water on luminous bacteria (SEAFDEC/AQD, 2001-2003)
 5. Screening of *Vibrio harveyi* bacteriophage for controlling luminous disease in marine shrimp hatchery (Samutsakhon Coastal Aquaculture Development Center [SCADC], Thailand, 2001-2003)
 6. Development of immuological indices for monitoring health status in *Penaeus monodon* (SEAFDEC/AQD, 2001-2003)
- D. Establishment of Evaluation Methods for Residual Chemicals in Aquaculture Products**
1. Establishment and monitoring on antimicrobial usage in shrimp aquaculture (SCADC, Thailand, 2001-2003)
 2. Detection of pesticide residues in aquaculture products (SEAFDEC/AQD, 2000-2003)
 3. Detection of antibiotic residues in aquaculture products (SEAFDEC/Marine Fisheries Research Department, 2002-2003)

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